Molecular Autopsy in Sudden Infant Death Syndrome (SIDS) and Sudden Unexplained Death (SUD) in the Young

Dissertation

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SUMMARY

Sudden and unexpected death of a previously healthy infant, adolescent or young adult is a tragic and distressing event for those left behind. In the majority of these cases the cause of death can be resolved by a comprehensive medicolegal investigation, including autopsy. However, approximately 30% of all individuals younger than 40 years of age remain unexplained after standard forensic examinations and are therefore termed as autopsy-negative sudden unexplained death (SUD) cases. In addition, infant deaths under one year of age are grouped into a separate category known as sudden infant death syndrome (SIDS).

For many SIDS/SUD cases, there are no apparent warning signs during lifetime and sudden death is often the first manifestation of an undetected genetic disease. It has been estimated that 10 to 30% of all SIDS/SUD cases carry sequence variants in known cardiac genes causing lethal cardiac arrhythmias in an otherwise morphological normal heart. Cardiomyopathies are typically characterized by a structural and functional abnormal heart muscle. Initial slight phenotypic alterations however might not be visible at autopsy, making a proper diagnosis difficult. Channelopathies are mainly caused by the dysfunction of transmembrane ion channels affecting the heart rhythm and cardiac electrical conduction.

Technical advances in high-throughput sequencing approaches facilitate the investigation of a large number of genes in a cost- and time-efficient manner. Therefore, whole-exome sequencing (WES) represents one of the most promising and powerful tools to elucidate potential death-causing mechanisms in absence of a clear phenotype in sudden death cases.

In a proof-of-concept study, five representative SUD cases were genetically investigated to test WES on post-mortem bad DNA quality samples. We focused on 184 cardiac disease-associated genes and were able to show that WES is a feasible option for this kind of samples and that post-mortem molecular autopsy can help to identify underlying cardiac diseases.

In the second part of the thesis, WES sequencing and analysis has been performed in two large sudden death cohorts composed of 161 SIDS and 34 SUD cases. By improving the filtering strategy and developing an own scoring system for the variant classification, likely pathogenic variants were identified in 20% of the SIDS cohort and in 29.4% of the SUD cohort. Interestingly, the diagnostic success was very high in SUD individuals younger than 18 years of age, strongly recommending a molecular autopsy examination of all sudden death cases in children and young adults.

In the third part of the thesis, a representative electrophysiological analysis was performed for one rare variant identified within the exome analysis of one SIDS victim. The heterozygous missense variant in the voltage-gated sodium channel β-subunit encoding gene *SCN1B* induced a loss-of-function of $Na_v1.5$ channels in the whole-cell patch-clamp experiment, indicating that this variant might have contributed to the vulnerability of the five-month old male SIDS infant.

We conclude that post-mortem molecular autopsy investigations in sudden death victims are very helpful in discovering underlying pathogenic mechanisms which have contributed to a sudden death event, mainly in young SUD victims. Additionally, genetic screening of the deceased has a significant impact on affected living family members in whom death-predisposing disorders might have remained unrecognized so far.

ZUSAMMENFASSUNG

Der plötzliche und unerwartete Tod eines scheinbar gesunden Säuglings oder jungen Menschen stellt ein schwer verkraftbares und belastendes Ereignis für die Angehörigen dar. Bei der Mehrheit dieser Todesfälle kann die Todesursache anhand einer umfassenden, gerichtsmedizinischen Untersuchung, bestehend aus einer Obduktion sowie histologischen, toxikologischen und mikrobiologischen Analysen, geklärt werden. Dennoch bleibt die Todesursache bei ungefähr einem Drittel dieser Todesfälle offen. Der plötzliche Säuglingstod (sudden infant death syndrome, SIDS) umfasst alle ungeklärten Todesfälle im ersten Lebensjahr und gilt als häufigste Todesursache bei Säuglingen in Industrienationen. Ungeklärten Todesfälle bei Kindern, Jugendlichen und jungen Erwachsenen bis zum vierzigsten Lebensjahr werden unter dem Begriff „plötzlicher, ungeklärter Tod“ (sudden unexplained death, SUD) zusammengefasst. Sowohl bei SIDS wie auch bei SUD wird vermutet, dass ungefähr 10 bis 30% der Betroffenen eine zuvor nicht diagnostizierte genetische Herzerkrankung hatten, bei welcher der plötzliche Tod oft ohne vorherige Warnsignale aufgetreten ist.

Die häufigsten vererbbaren Herzerkrankungen sind Kardiomyopathien und kardiale Ionenkanalerkrankungen. Kardiomyopathien sind eine heterogene Gruppe von Herzmuskelerkrankungen, die mit mechanischen und elektrischen Funktionsstörungen einhergehen und üblicherweise, oft aber erst im fortgeschrittenen Verlauf der Erkrankung, eine Verdickung oder Erweiterung der Herzkammern hervorrufen. Kardiale Ionenkanalerkrankungen sind charakterisiert durch episodisch auftretende Erregungsstörungen der Herzmuskultur, die lebensgefährliche Herzrhythmusstörungen auslösen können. Da viele dieser Herzerkrankungen zu keinen oder nur minimalen strukturellen Herzmuskelveränderungen führen, sind sie postmortal oft nur schwer erkennbar.

Technische Weiterentwicklungen in der Hochdurchsatzsequenzierung ermöglichen eine kostengünstige und schnelle Sequenzierung einer grossen Anzahl an Genen oder des gesamten Genoms. Die Sequenzierung des Exoms (whole-exome sequencing, WES), welches die Gesamtheit aller proteinkodierenden Bereiche umfasst, stellt eine der vielversprechendsten Methoden dar, um komplexe genetische Erkrankungen, wie zum Bespiel SIDS und SUD, aufzudecken.

Im ersten Teil dieser Dissertation wurden in einem Pilotprojekt fünf SUD Fälle mittels WES molekulargenetisch untersucht. Ziel dieses Projektes war zu testen, ob postmortale DNA Proben, welche oft von schlechter Qualität sind, für diese Sequenzierungsmethode geeignet sind. In einer

ersten Auswertung wurden 184 Gene untersucht, die mit Herzerkrankungen assoziiert sind. In drei der fünf Fälle konnten genetische Herzerkrankungen diagnostiziert werden. So konnte gezeigt werden, dass WES eine praktikable Methode für diese Art von DNA-Proben darstellt und dass eine postmortale molekulargenetische Untersuchung zur Aufdeckung von genetischen Herzerkrankungen beitragen kann.

Basierend auf den erarbeiteten Sequenzierungs- und Auswertungsmethoden wurden im zweiten Teil dieser Dissertation 161 SIDS und 34 SUD Fälle molekulargenetisch untersucht. Eine Verbesserung der Filterstrategie und die Entwicklung eines Punktesystems für die Beurteilung der Genvarianten führte zur Aufklärung von 20% der SIDS und in 29.4% der SUD Fälle. Die hohe Aufklärungsrate in Kindern und Jugendlichen deutet darauf hin, dass speziell in dieser Altersklasse eine postmortale molekulargenetische Untersuchung massgeblich zur Aufklärung der Todesursache beitragen kann.

Im dritten Teil dieser Dissertation wurde eine Genvariante, welche in den Exom-Daten eines im Alter von fünf Monaten verstorbenen Säuglings entdeckt worden ist, mittels elektrophysiologischer Analysen funktionell untersucht. Der Patch-Clamp-Versuch zeigte, dass die heterozygote Variante im *SCN1B*-Gen, welches für die β-Isoform der spannungsabhängigen Natriumkanäle kodiert, einen Funktionsverlust erzeugt und somit zur Vulnerabilität des Säuglings beigetragen haben könnte.

Zusammenfassend konnte in dieser Dissertation gezeigt werden, dass eine postmortale molekulargenetische Autopsie vor allem bei Kindern und Jugendlichen eine wertvolle Ergänzung zur gerichtsmedizinischen Untersuchung darstellt und bis anhin unerkannte Herzerkrankungen aufdecken kann. Zusätzlich sind diese genetischen Befunde wichtig für betroffene Familienmitgliedern, die ebenfalls eine solche Herzerkrankung haben könnten.

TABLE OF CONTENTS

ABBREVIATIONS

AAP	American Academy of Paediatrics
ACMG	American College of Medical Genetics and Genomics
ARVC	Arrhythmogenic right ventricular cardiomyopathy
BrS	Brugada syndrome
CAD	Coronary artery disease
CPVT	Catecholaminergic polymorphic ventricular tachycardia
CNV	Copy number variation
DNA	Deoxyribonucleic acid
DCM	Dilated cardiomyopathy
ECG	Electrocardiography
EmPCR	Emulsion PCR
HCM	Hypertrophic cardiomyopathy
ICD	Implantable cardioverter defibrillator
InDels	Insertions/deletions
LQTS	Long QT syndrome
lncRNA	Long non-coding RNA
LVNC	Left ventricular non-compaction cardiomyopathy
MAF	Minor allele frequency
MPS	Massive parallel sequencing
MRI	Magnetic resonance imaging
mtDNA	Mitochondrial DNA
miRNA	Micro RNA
mRNA	Messenger RNA
NGS	Next-generation sequencing
NICHD	National Institute of Child Health and Human Development
piRNA	Piwi-interacting RNA
PPCM	Peripartum cardiomyopathy
QTc	Corrected QT interval
RNA	Ribonucleic acid
SCD	Sudden cardiac death
SD	Sudden death
SIDS	Sudden infant death syndrome
siRNA	Short interfering RNA
SNCD	Sudden non-cardiac death
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
SQTS	Short QT syndrome
SUD	Sudden unexplained death
SUDEP	Sudden unexpected death in epilepsy
VUS	Variant of unknown significance
WES	Whole-exome sequencing
WGS	Whole-genome sequencing
ZIFM	Zurich Institute of Forensic Medicine

I. INTRODUCTION

1.1. Sudden unexplained death in infants and young adults

Sudden and unexpected death of a previously healthy infant or young family member is a tragic and distressing event for those left behind. Sudden death (SD) is defined as a witnessed, natural and unexpected death occurring within 1h after onset of acute changes in cardiovascular status, leading to cardiac arrest in a previously healthy person[1]. If the sudden death event remains unwitnessed, the person has to be seen alive and well within 24h before being found dead.

In the majority of these cases, the underlying causes of death can be resolved by a comprehensive medicolegal investigation, including autopsy[2, 3]. Based on the post-mortem examination, SD cases are then divided into sudden cardiac death (SCD) or sudden death due to non-cardiac causes (SNCD), such as pulmonary embolism or metabolic diseases (Figure 1)[4]. However, approximately 30% of all sudden death cases in individuals younger than 40 years of age remain unexplained after standard forensic investigation and are therefore termed as autopsy-negative sudden unexplained death (SUD)[5]. Sudden unexplained deaths of infants under one year of age are grouped into a separate category known as sudden infant death syndrome (SIDS)[6]. Despite of intensive scientific research to identify underlying pathophysiological mechanisms leading to sudden death events, the cause of death still remains unknown in the majority of SIDS and SUD cases.

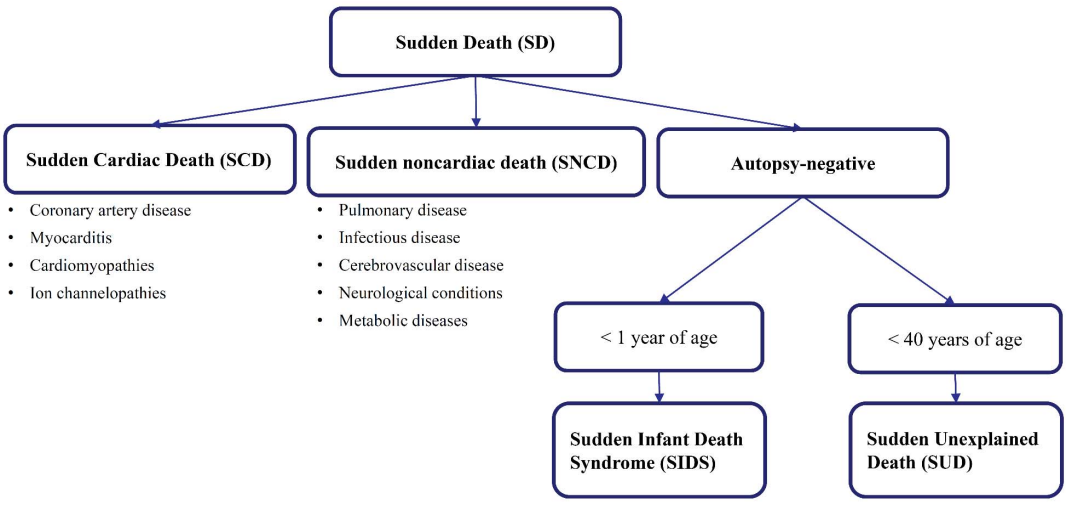


Figure 1 Classification of sudden death in the young (0-40 years of age).

1.2. Sudden infant death syndrome (SIDS)

1.2.1. Definition of SIDS

The term "sudden infant death syndrome (SIDS)" was first proposed in 1969 at the *Second International Conference on Causes of Sudden Death in Infants* near Seattle, Washington[7]. Although relatively consistent clinical, epidemiological, and pathological features were already known, the definition of SIDS was formulated as general as possible, with the intention to avoid the exclusion of cases which might later be diagnosed as the same mechanism or cause[8]. Therefore, the first SIDS definition, designated as the 1969 definition, was as follows:

> *"The sudden death of any infant or young child, which is unexpected by history, and in which a thorough post-mortem examination fails to demonstrate an adequate cause for death"*[7].

Despite the claim for a more specific definition, it took 20 years until the SIDS definition was reconsidered in 1989. Examinations of retrospective SIDS cases had strengthened the evidence of a similar age distribution in sudden death cases with a highest peak between two and four months of age, an association with minor viral inflammation, prematurity, and social disadvantage[9]. In 1991, an age limitation below one year of age was included into the definition[10]. In 1992, at the *SIDS International Meeting* in Sydney, Australia, a separation of SIDS cases into typical and atypical groups was proposed[11]. Although the proposal was not accepted at that time point, the need of a re-evaluation became more and more evident in the following years. In 2004, a meeting was held in San Diego, California, at which a consortium decided that the definition of SIDS needs to be more precise, including additional specifications for diagnosis and improved autopsy investigation guidelines. The following definition has been agreed as the SIDS San Diego definition which is still valid today:

> *"SIDS is defined as the sudden unexpected death of an infant younger than 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy, and review of the circumstances of death and the clinical history"*[6].

In addition, SIDS cases are subdivided into three different categories, namely SIDS I and II, and an additional unclassified category (Table 1).

	Category SIDS I	Category SIDS II
General	*Category I includes infant deaths that meet the requirements of the general definition and also all the following requirements.*	*Category II includes infant deaths that meet category I criteria except for one or more of the following alternatives:*
Clinical history	• More than 21 days and < 9 months of age. • Normal clinical history, including term pregnancy (gestational age of ≥ 37 weeks). • Normal growth and development. • No similar deaths among siblings, close genetic relatives, or other infants in the custody of the same caregiver.	• Age range outside that of category I. • Neonatal or perinatal conditions (for example, those resulting from preterm birth) that have resolved by the time of death. • Abnormal growth and development not thought to have contributed do death. • Similar deaths among siblings, close relatives, or other infants in the custody of the same caregiver that are not considered suspect for infanticide/recognized genetic disorders.
Circumstances of death	• Investigation of the various scenes where incidents leading to death might have occurred and determination that they do not provide an explanation of the death. • Found in a safe sleeping environment, with no evidence of accidental death.	• Mechanical asphyxia or suffocation caused by overlaying not determined with certainty.
Autopsy	• Absence of potentially fatal pathological findings. Minor respiratory system inflammatory infiltrates are acceptable; intrathoracic petechial hemorrhage is a supportive but no obligatory or diagnostic finding. • No evidence of unexplained trauma, abuse, neglect, or unintentional injury. • No evidence of substantial thymic stress effect. Occasional "starry sky" macrophages or minor cortical depletion is acceptable. • Negative results of toxicological, microbiologic, radiologic, vitreous chemistry, and metabolic screening studies.	• Marked inflammatory changes or abnormalities not sufficient to be unequivocal causes of death.

Table 1 SIDS classification according to the San Diego definition[6].

The San Diego definition consists of a general definition and furthermore subdivides the SIDS cases into category I and II, and an additional unclassified category. The unclassified category describes a collective term with respect to cases without performed autopsy or where the requirements for category I and II are not met. Hence this category is not listed in the table.

Although the definition of SIDS has been adjusted continuously until the final version in 2004, SIDS still remains a diagnosis by exclusion, based on the subjective interpretation of the responsible pathologist[12, 13]. Beside this, some infant death cases might have been misdiagnosed as SIDS due to the difficulties to distinguish a natural death from a unnatural death due to suffocation, often not detected during forensic autopsy investigations[14].

1.2.2. Incidence rate of SIDS

The incidence rate of SIDS varies considerably among countries and populations. The lowest number of SIDS cases are reported in the Netherlands and in Japan (0.08 - 0.1 death cases per 1'000 live births) and the highest number of cases in New Zealand (0.77 death cases per 1'000 live births)[15, 16]. An increased SIDS rate is mainly observed in infants from indigenous populations such as African Americans, American Indians, the Aboriginal Australians, Maoris, and those of mixed ancestry in Cape Town, South Africa[17]. In Switzerland, the incidence rate of SIDS has remained constant with 0.1 death cases per 1'000 live births after the implementation of the San Diego definition in 2004 (Figure 2).

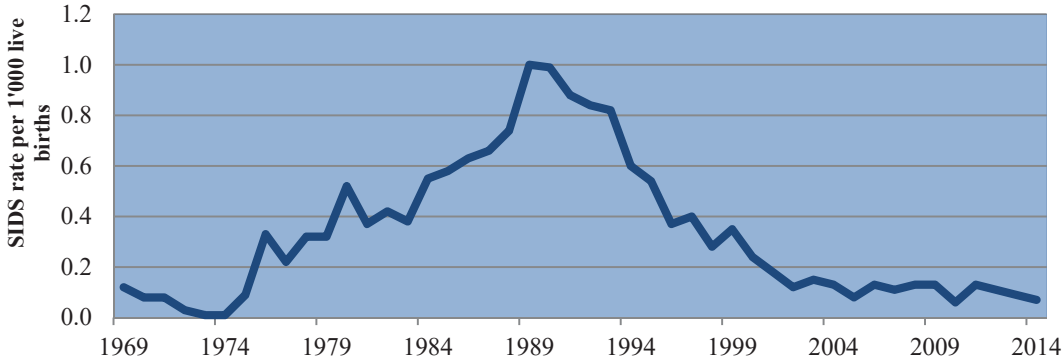


Figure 2 SIDS incidence rate in Switzerland from 1969 to 2014 provided by the "Bundesamt für Statistik (BFS)".
In Switzerland, unexplained infant death cases were recorded since the introduction of the first SIDS definition in 1969. As in other countries, the incidence rate of SIDS continuously increased in the following years. The sharp decrease in the SIDS rate in the 1990ies correlates with the introduction of international "back-to-sleep" recommendations to place infants in a supine sleeping position. Since 2004, Switzerland has a stable SIDS incidence rate of approximately 0.1 death cases per 1'000 live births.

The worldwide decline of SIDS cases in the 1990ies is basically explained by the changes in the SIDS definition and the recognition of several environmental risk factors. The "back-to-sleep" recommendations published by the American Academy of Pediatrics (AAP) and the National Institute of Child Health and Human Development (NICHD) identified the prone sleeping position of an infant as one of the major risk factors in SIDS. Therefore, parents and caregivers were sensitized to place their infants in a safe sleeping position by avoiding the prone position[18, 19]. Despite this strong decrease of SIDS cases since 1989, SIDS still remains a major cause of infant mortality in many countries[20].

1.2.3. Triple risk hypothesis

The occurrence of SIDS is described by a complex multifactorial event including a combination of genetic and environmental risk factors. In 1994, Filiano and Kinney proposed a triple risk

hypothesis where three overlapping classes of SIDS-associated risk factors contribute to the event of death, namely (1) a vulnerable infant, (2) a critical developmental period in homeostatic control, and (3) exogenous stress factors[21]. According to this hypothesis, an infant will suddenly die only when all three risk factors are present at the same time point (Figure 3).

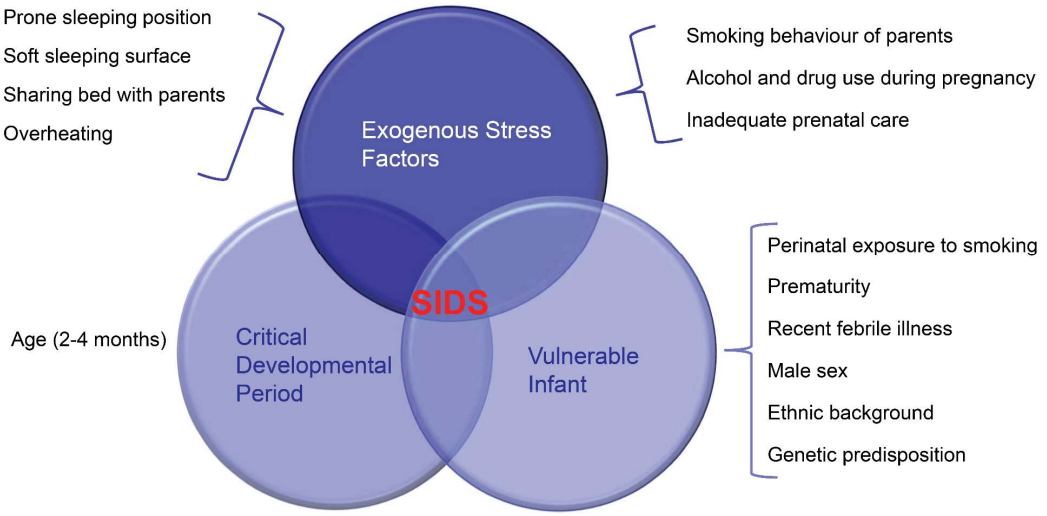


Figure 3 Triple risk model in SIDS.

SIDS might occur in a situation when several risk factors are present simultaneously. These risk factors include a critical developmental period with a highest risk between two to four months of age, exogenous stress factors such as the prone sleeping position, sharing bed with parents or alcohol and drug use during pregnancy, and a vulnerable infant caused by prematurity, recent illness, or genetic predispositions.

The critical developmental period spans the first 12 months of an infant's life with the majority of SIDS deaths occurring between 2 and 4 months after birth[22]. Multiple studies have identified this period as a time when the brain of an infant is undergoing rapid and extensive physiological changes[23]. Inflammation and inflammatory cytokines, as well as abnormalities in neurotransmitters, could lead to disturbances in the homeostatic response of the respiratory network, placing an infant to life-threatening situations in combination with additional stressors[24]. Exogenous or environmental risk factors are related to the sleeping circumstances of an infant such as the prone sleeping position, soft sleeping surface, sharing bed with parents, or overheating. When the prone sleeping position had been recognized as a major risk for SIDS, attention turned towards factors that can affect normal arousal and autoresuscitation while sleeping on the stomach. Such factors include asphyxia due to airway compression or rebreathing of exhaled gases in the face-down position, impaired cardiorespiratory regulation related to heat stress, and impaired heat loss with subsequent hyperthermia when the face is pressed against bedding[15]. Maternal alcohol consumption and/or postnatal exposure to smoking are additional environmental risk factors, as these factors have a direct effect on neurotransmitter systems in the

developing brain of an infant[25-27]. The vulnerability of an infant is in addition affected by prematurity, recent illness, male sex, ethnic background, and genetic predisposition[15].

1.2.4. Genomic risk factors in SIDS

In the last years, numerous genetic case-control studies have investigated several pathophysiological mechanisms possibly contributing to the sudden death event of an infant[28]. Based on the hypothesis that SIDS is a homeostatic disorder, most of these studies have focused on genes involved in (1) the central nervous system and brain development, (2) immune system dysfunction, (3) metabolism and energy pathway, (4) nicotine response and (5) respiratory dysfunction (Table 2)[29, 30]. Typically, specific genotypes in genes within these pathways were reported to occur significantly more often in SIDS cases compared to age -and gender-matched controls. Although such sequence alterations cannot clearly be correlated to physiological dysfunction or lethal diseases, they may display a genetic determinant predisposing an infant to an increased vulnerability[31].

Pathway	Investigated genes	References
Central nervous system and brain development	*APOE, AQP4, COMT, DBH, FEV, MAOA, PACAP, PHOX2B, TH01, TPH2, TPSYL1, 5-HTT, 5-HT1A, 5-HT2A*	32-50
Immune system dysfunction	*C4A, C4B, IL-6, IL-10, MBL2, TGFβ3, TNFα, VEGF*	51-58
Metabolism and energy pathway	*ACADM, ACADS, CPT1A, CPT2, GK, G6PC, G6PT1,* mtDNA	59-61
Nicotine response	*CYP1A1, FMO3, GSTT1*	62, 63
Respiratory dysfunction	*P2RY1, SSTR2*	64

Table 2 Summary of investigated genes with possible predisposing effects within different pathways.

Autonomic function and neurotransmission

The medullary serotonin or 5-hydroxytryptamine (5-HT) system modulates and integrates several autonomous homeostatic functions like gasping and ventilation, thermoregulation, autonomic control, response to carbon dioxide and oxygen, arousal from sleep, and hypoxia induced plasticity[31]. Therefore, an impairment of the system might be involved in the pathophysiological pathway of SIDS. Two regions in the serotonin transporter gene (*5-HTT*) were investigated in several SIDS cohorts of different ethnicities[37-42], but with controversial findings, concluding that *5-HTT* gene variants are unlikely to play a major role in the pathogenesis of SIDS. Various other genes involved in autonomous regulation and brain development such as the monoamine oxidase A (*MAOA*)[34-36], tryptophan hydroxylase 2 (*TPH2*)[39], or tyrosine hydroxylase (*TH01*)[48-50], have been examined in different SIDS cohorts, but only little evidence was found that variants within these genes play a critical role during acute SIDS episodes[65].

Immune system dysfunction

Based on post-mortem examination reports, many SIDS infants showed signs of slight infections in the upper respiratory tract, digestive tract, nervous system and blood. These findings suggested that at least a subset of the infants that later die of SIDS are more vulnerable to simple infections and that an activated immune system may be involved in the SIDS pathogenesis[58]. Genetic studies investigated several genes encoding some of the key modulators in the immune system such as components of the complement system of innate immunity, interleukins and cytokines, but only a few of them reported positive associations[58].

Metabolic and energy pathway

Inborn dysfunctions of metabolism account for approximately 1-2% of sudden death cases during the first year of life[29]. The majority of these cases had variants in the medium-chain acyl-CoA dehydrogenase encoding gene *ACADM* which catalyzes the first step in the β-oxidation of fatty acids[66]. Furthermore, as low blood glucose concentrations can lead to death, it was hypothesized that mutations in the glucokinase and glucose-6-phosphatase encoding genes *GK* and *G6PC* may be involved in SIDS[59, 60]; however none of the investigated polymorphisms were associated with SIDS. Another interesting pathway which might be impaired in a subset of SIDS cases is the energy production of the mitochondria[67]. Mitochondrial diseases tend to affect tissue with high energy demands, including the brain, muscle, heart, and endocrine system[68]. As some of the infants have been reported with apathy, increased sleepiness, and a lower activity score, it has been assumed that mitochondrial DNA (mtDNA) variants involved in a decreased ATP production might contribute to SIDS[29]. But so far, no predominant mtDNA mutations were reported to be associated with SIDS.

Nicotine response

While smoking during pregnancy is a well described risk factor for SIDS, only two genetic studies have examined the potential association between SIDS and polymorphisms in nicotine metabolizing enzymes. A common variant in the flavin-monooxygenase 3 encoding gene *FMO3* was over-represented in SIDS cases whose mothers reported heavy smoking during pregnancy highlighting the potential interaction between genetic vulnerability and environmental insult in SIDS pathogenesis[62]. In contrary, polymorphisms in the nicotine metabolizing enzymes glutathione s-transferase theta 1 and cytochrome P450 encoding genes *GSTT1* and *CYP1A1* are not responsible for an increased SIDS risk[63].

Ion channelopathies and cardiomyopathies

Already more than 30 years ago, electrocardiographic investigations of infants that later died of unknown causes suggested that an undetected cardiac channelopathy might have contributed to the sudden death event in some of the infants[69, 70]. In 1998, a huge prospective study of over 34'000 infants showed that a prolongation of the QT interval in the first week of life is strongly associated with SIDS[71]. Later, spontaneous mutations in the $Na_v1.5$ cardiac sodium channel encoding gene *SCN5A* were identified in an infant who nearly died of SIDS and in whom the long QT syndrome was diagnosed, providing a first "proof of concept" of cardiac ion channelopathies as a cause of SIDS[72]. Since then, mutations in several cardiomyopathy and channelopathy disease-associated genes have been identified in different SIDS cohorts (Table 3), however, many of these studies were limited to single genes due to limited sequencing technologies at that time.

Pathway	Investigated genes	Variants	References
Cardiomyopathies	*MYBPC3*	S217G, D610H, A833T, E907K*, P910T, I1131T, E1179K, A1194T	70, 73
	MYH6	A1004S	73
	TNNI3	S166F	73
Cardiac ion channelopathies	*CAV3A*	V14L, T78M, L79R	74-76
	GJA1	E42K, S272P	77
	GPD1L	E83K, I124V, R273C	78
	KCND3	S530P	79
	KCNE1	T20I	80
	KCNE2	Q9E, V14I	76, 81
	KCNH2	K101E, R148W R273Q, V279M, G294V, N558K, R752Q, R885C, T895M, K897T, P926AfsX14, R954C, S1040G, P1157L	76, 80, 82, 83
	KCNJ8	E332del, V346I	84
	KCNQ1	H105L, P117L, I274V, G460S, K598R, T600M, G626S	76, 80, 85-87
	RYR2	R2267H, S4565R	88
	SCN1B	R214Q	89
	SCN3B	V36M, A54G	90
	SCN4B	S206L	90
	SCN5A	S16L, S524Y, F532C, L567Q, delA586/L587, R680H, W822X, S941N, A997S, G1084S, S1103Y, R1193Q, A1330P, S1333Y, R1826H,	72, 87, 91-97
	SNTA1	T262P, S287R, T372M, G460S	98

Table 3 Summary of identified genetic alterations in cardiac disease-associated genes in SIDS cohorts.

1.3. Sudden unexplained death (SUD) in adolescents and young adults

Sudden death in children, adolescents, and young adults between 1 to 40 years of age has received increasing awareness in the public, mainly due to death cases in famous top athletes. The vast majority of sudden death cases are considered due to cardiac causes, whereas NCSD such as pulmonary embolism, asthma or metabolic diseases account for only 15% of death cases (Figure 4)[4]. SCD is one of the most common causes of death in developed countries and an estimated 300'000 to 400'000 individuals die suddenly each year in the United States[99]. The majority of these SCD events occur in elderly persons and are caused by coronary artery disease (CAD). In comparison, sudden cardiac death in individuals younger than 40 years of life is relatively uncommon, with an incidence rate of 1.3 to 8.5 per 100'000 death cases per year[2]. In young athletes, high risk groups are males and African-Americans and the majority of reported death cases occurred during participation in basketball and football games[100]. Unfortunately, the epidemiology of sudden death in the young is often less apparent and approximately 30% of all sudden death victims in this age category have no definite cardiac etiology identified after autopsy investigations and are therefore termed as SUD cases (Figure 4 and Figure 5)[5].

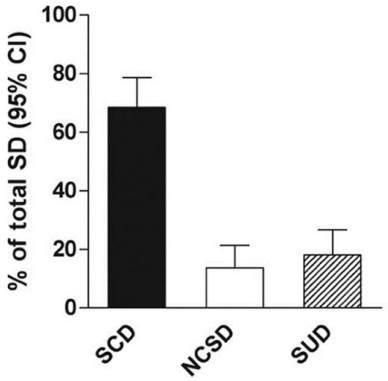


Figure 4 Distribution of cases of SD ≤ 40 years in the general population, n = 486 (copied from Van der Werf *et al.*)[4].
SD = sudden death, SCD = sudden cardiac death, NCSD = noncardiac sudden death, SUD = sudden unexplained death.

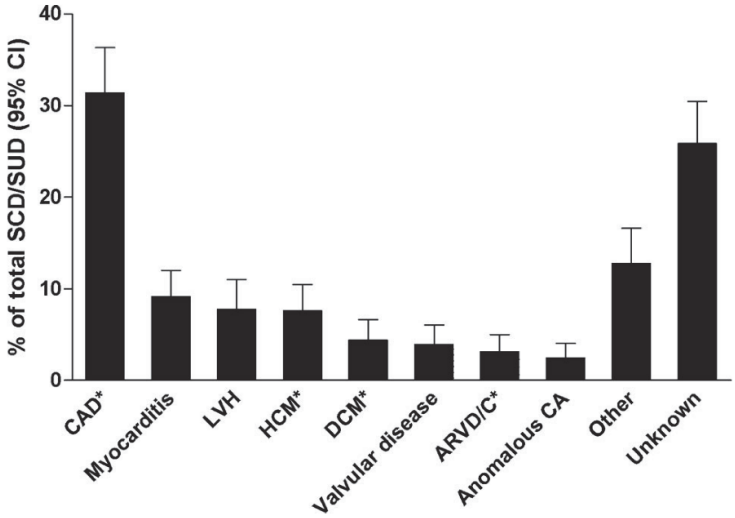


Figure 5 Distribution of causes of SCD ≤ 40 years in the general population (copied from Van der Werf *et al.*)[4]

Displayed are the weighted mean proportions (95% CI) per cause of death as a proportion of the total number of SCD and SUD cases. n = 1537, CAD = coronary artery disease, LVH = left ventricular hypertrophy, HCM = hypertrophic cardiomyopathy, DCM = dilated cardiomyopathy, ARVD/C = arrhythmogenic right ventricular dysplasia/ cardiomyopathy, SCD = sudden cardiac death, SUD = sudden unexplained death.

The exact incidence of SUD in the younger population remains unclear in many countries, including Switzerland, mainly due to the fact that these death cases are not registered as SUD but as natural death case in the forensic setting[101, 102].

For many SCD/SUD cases, there are no apparent warning signs and sudden death is often the first manifestation of an undetected genetic cardiac disease causing lethal arrhythmias in a morphological normal heart[76, 103]. Cardiomyopathies are typically characterized by a structural and functional abnormal heart muscle; however, initial slight phenotypic alterations might not be visible at autopsy, making a proper diagnosis difficult[104]. Channelopathies are primarily caused by the dysfunction of transmembrane ion channels, affecting the heart rhythm and cardiac electrical conduction[105].

1.4. Cardiomyopathies

The heart is an extremely specialized muscle with four chambers that continuously pump blood through two independent vascular systems into the body and the lungs[106]. During diastole (cardiac relaxation) blood flows from the atria into the muscular ventricles and the right and the left ventricles pump blood into the pulmonary and system circulations during systole (cardiac contraction). Although both ventricles pump the same stroke volume, the right ventricle has a more thinner wall compared to the left ventricle due to the lower resistance of the pulmonary vasculature.

Morphological abnormalities in the structure of the cardiac muscle have been defined by the American Heart Association as follows: "*Cardiomyopathies are a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction that usually (but not invariably) exhibit inappropriate ventricular hypertrophy or dilatation and are due to a variety of causes that frequently are genetic. Cardiomyopathies either are confined to the heart or are part of generalized systemic disorders, which may lead to cardiovascular death or progressive heart failure-related disability*"[107].

Cardiomyopathies are divided into primary cardiomyopathies, which affect the heart alone and secondary cardiomyopathies, which are the result of a systemic illness affecting many other organs of the body such as endocrine, neurological, and autoimmune disorders[108]. The different types of primary cardiomyopathies are classified based on anatomical and physiological features into arrhythmogenic right ventricular cardiomyopathy (ARVC), dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), and left ventricular non-compaction cardiomyopathy (LVNC) (Table 4)[109]. A common feature for all cardiomyopathies is that they are genetically very heterogeneous, with incomplete penetrance and variable expressivity[110]. Additionally, there are

substantial genetic and phenotypic overlaps between the different classifications complicating a precise clinical diagnosis[111].

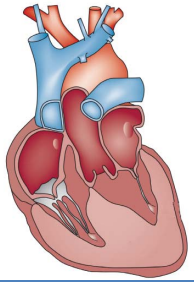
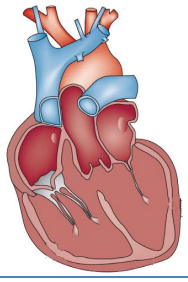
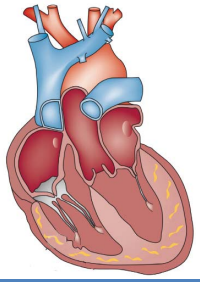
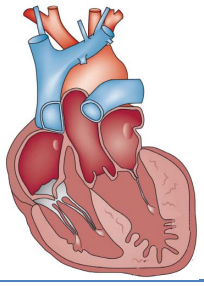
	ARVC	DCM	HCM	LVNC
Clinical characterization	• Myocyte loss and fibro-fatty tissue replacement of the right ventricle • Electrical instability 	• Left ventricular enlargement or dilatation • Systolic dysfunction 	• Thickening of the left ventricle with a small ventricular cavity dimension • Myocardial fiber disarray 	• Spongy myocardium • Trabeculations 
Prevalence	Between 1-2.5:2000	1:250	1:500	1:10’000
Age of onset	Around 30 years of age	Adult onset	Adolescence or young adulthood	Children or adults
Genetics	• 13 genes • Mutations in *DSG2*, *DSP*, and *PKP2* account for 40-50% of cases	• More than 50 genes • Mutations in *LMNA*, *MYH6*, *MYH7*, *MYPN*, and *TTN* account for 30% of cases	• More than 20 genes • Mutations in *MYH7*, *MYBPC3*, *TNNT2*, and *TNNI3* account for 40-60% of cases	• More than 40 genes • Mutations in *ACTC1*, *MYBPC3*, *MYH7*, *LMNA*, *TNNT2*, *TNNI3*, and *TPM1* account for 30% of cases
Mutation detection rate*	• ~60%	• ~50%	• 20-60%	• 35-40%
Therapy and surveillance	• Lifestyle modifications • Particularly exercise restriction • Antiarrhythmic medications • Catheter ablation • ICD implantation	• Pharmacologic therapy • Pacemakers or ICD • Management of pregnancy-associated DCM	• Avoidance of competitive endurance training, burst activities, intense isometric exercise, dehydration, and medications that decrease afterload • Pharmacologic therapy • Pacemakers or ICD	• Pharmacologic therapy • Pacemakers or ICD

Table 4 Classification of cardiomyopathies[110].

ARVC = arrhythmogenic right ventricular cardiomyopathy, DCM = dilated cardiomyopathy, HCM = hypertrophic cardiomyopathy, LVNC = left ventricular non-compaction, ICD = implantable cardioverter defibrillator. * = percentage of detected variants in genetic screening.

1.4.1. Arrhythmogenic right ventricular cardiomyopathy (ARVC)

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is characterized by myocyte loss and fibro-fatty replacement of the right ventricular myocardium and electrical instability resulting in life-threatening ventricular arrhythmias and slowly progressive ventricular dysfunction[112-114]. The estimated prevalence of ARVC in the general population ranges from one in 2000 to one in 5000 individuals and it accounts for approximately 10-30% of cases of SCD in young adults and athletes[115, 116]. Especially ARVC patients who participate in competitive sport exercises have a five-fold increased risk of SCD compared to non-athletes[117]. ARVC affects more men than women, with an approximate ratio of 3:1, and first symptoms usually arise from the 2nd to the 4th decade of life[118].

Clinical characteristics

As ARVC progressively develops, structural changes may be absent or confined to a localized region of the right ventricle in the early stage of the disease (Figure 6A). Hallmark of ARVC is the so-called "triangle of dysplasia", including the right ventricular outflow tract, apex, and anterior infundibulum[112]. Histological examinations typically present with transmural fibro-fatty replacement of right ventricular epicardium or mid-myocardium (Figure 6B)[119]. With disease progression, further involvement of the right ventricular free wall, and left ventricular involvement can occur[114].

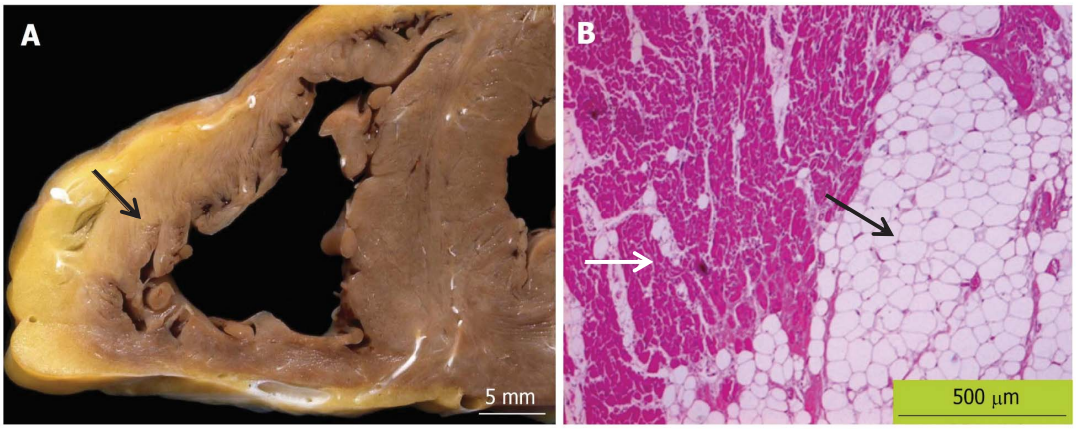


Figure 6 Typical pathological findings in ARVC (copied from Saguner *et al.*)[120].

A) Macroscopic findings in a patient with ARVC. The myocardium of the right ventricular free wall is partially replaced by fibro-fatty tissue (black arrow) that typically begins in the epicardial region and expands transmurally at later stages. B) Endomyocardial biopsy from a patient with ARVC demonstrating fatty (black arrow) replacement of the right ventricular myocardium. Strands of myocardium are still visible (white arrow; heidenhain trichrome staining, magnification 60x).

The most frequent symptoms in ARVC are palpitations, dizziness, syncope, chest pain, and dyspnea. The diagnosis of ARVC is currently based on the presence of major and minor standardized "Task Force Criteria" including clinical and family history, physical examination, chest radiography, 12-lead ECG, and two-dimensional echocardiography[121]. Selected patients in whom non-invasive assessment is inconclusive might require further examination by contrast-enhanced cardiac magnetic resonance (MRI), contrast angiography, and endomyocardial biopsy[122].

Beyond the classical form of ARVC, a biventricular and a left dominant form are now considered as different patterns of the wide spectrum of ARVC[116, 123]. According to current knowledge, only one extra-cardiac manifestation of ARVC exists, namely Naxos disease, characterized by additional wooly hair and palmoplantar keratoderma[124].

Genetic background

A familial history of ARVC is present in 30-50% of cases and ARVC is usually inherited in an autosomal dominant pattern[125]. The causal mechanism in ARVC involves defects at the intercalated discs of cardiomyocytes, which are composed of gap junctions and desmosomes and normally maintain mechanical and electrical connections for proper cardiac contraction[126]. Desmosomes are complex multiprotein structures and are both vital for cell-cell contact and cell signaling (Figure 7)[127].

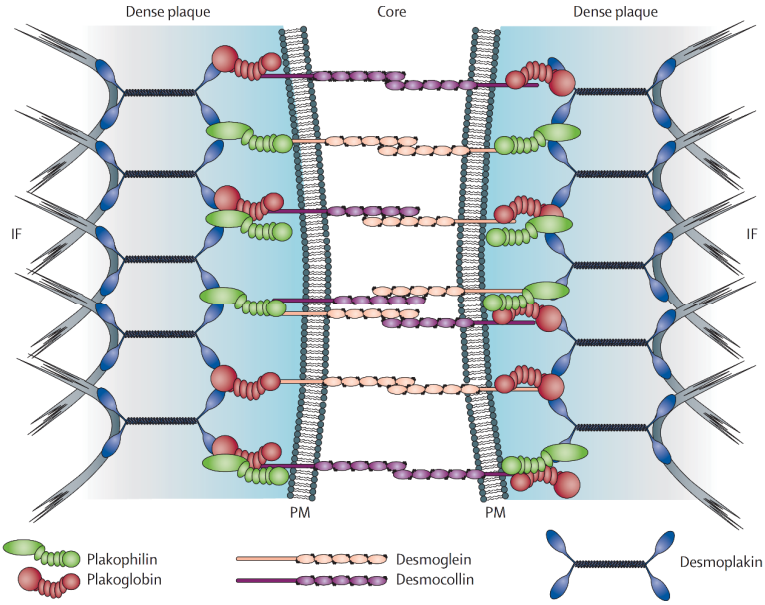


Figure 7 Intracellular mechanical junctions of the cardiomyocyte (copied from Basso *et al.*)[114].

There are three major groups of desmosomal proteins: transmembrane proteins (cadherins) including desmocollins and desmogleins; desmoplakin that binds directly to intermediate filaments; and linker proteins (armadillo family proteins including plakoglobin and plakophilins which mediate interactions between the desmsomal cadherin tails and desmoplakin. IF = intermediate filaments, PM = plasma membrane.

Desmosomal cadherins (desmoglein and desmocollin) are transmembrane proteins providing mechanical coupling of opposing cells[126]. Desmoplakin serves to anchor the desmosomal structure to the intermediate filaments of the cell and armadillo proteins are linkers between desmoplakin and the cadherin tails[128]. Mechanical uncoupling of desmosomes and adherens junctions is accompanied by cell death and regional fibrosis, which causes the monomorphic arrhythmias typically associated with ARVC[121]. In addition, electrical uncoupling based on gap junction remodeling may lead to significant activation delay, which increases the propensity of functional block and arrhythmia.

So far, mutations in five desmosomal genes and six non-desmosomal genes have been reported in ARVC patients (Table 5)[121]. Junction plakoglobin (*JUP*) was the first desmosomal gene to be associated with the classical autosomal dominant form of the disease[129]. On the basis of its mutation rate, desmoglein-2 (*DSG2*), plakophilin-2 (*PKP2*) and desmoplakin (*DSP*) belong to the so-called "3 big ARVC" genes, as about 90% of ARVC mutations are located in these genes[130]. In rare cases, ARVC has been related to mutations in non-desmosomal genes including alpha T-catenin (*CTNNA3*)[131], lamin A/C (*LMNA*)[132], cardiac ryanodine receptor 2 (*RYR2*)[133], transforming growth factors β-3 (*TGFβ3*)[134], and trans-membrane protein 43 (*TMEM43*)[135]. Comprehensive mutation screening of all known ARVC genes can detect causative variants in approximately 60% of patients[136].

Gene	Protein name	Protein localisation / Function	Estimated prevalence
CTNNA3	Catenin alpha 3	Cytoskeleton, links actin to sites of cell-cell contact	rare
DES	Desmin	Cytoskeleton, transduces contractile forces	rare
DSC2	Desmocollin-2	Desmosome, links desmosomes of neighbouring cells	**5-25%**
DSG2	Desmoglein-2	Desmosome, links desmosomes of neighbouring cells	rare
DSP	Desmoplakin	Desmosome, tethers intermediate filaments to desmosomal plaques	**1-16%**
JUP	Junction plakoglobin	Desmosome, links desmoplakin to desmosomal cadherins	rare
LDB3	LIM binding domain 3 (ZASP)	Cytoskeleton, clustering of membrane proteins	1%
LMNA	Lamin A/C	Nuclear envelope, stability of inner nuclear membrane, regulation of gene expression	rare
PLN	Phospholamban	Sarcoplasmic reticulum, protein binding	<1%
RYR2	Ryanodine receptor 2	Sarcoplasmic reticulum, ion channel activity	rare
PKP2	Plakophilin2	Desmosomal, links desmoplakin to desmosomal cadherins	**7-70%**
TGFβ3	Transforming growth factor β3	Cytokine involved in extracellular matrix deposition, cell adhesion and cellular signalling	rare
TMEM43	Transmembrane protein 43	*Area composita* protein, binds lamins and emerin	rare

Table 5 Genes associated with ARVC.

Treatment

The current strategies and therapies aim to minimize ventricular arrhythmias and delay the progression of disease by lifestyle modification, particularly exercise restriction, β-blocking medications, anti-arrhythmic medications, catheter ablation of ventricular arrhythmias, and ICD implantation[137].

1.4.2. Dilated cardiomyopathy (DCM)

The phenotypic characterization of dilated cardiomyopathy (DCM) is defined by systolic dysfunction, left ventricular enlargement, and a reduction in ventricular wall thickness[138]. Since a large number of cardiac and systemic diseases can cause systolic impairment, the clinical term "idiopathic DCM" is used for patient without any identifiable non-genetic causes of the disease[139]. The prevalence of DCM was originally coming from a study conducted in the years 1975-1984 in Olmsted County, MN, USA, that estimated the prevalence at 1:2700[140]. According to more recent studies, the prevalence of DCM is estimated at higher frequency of around 1:250[141]. DCM commonly presents in the 4th to 6th decades of life, but onset in infancy and early childhood has been reported as well[142]. Although DCM may be asymptomatic for months to years, it almost always presents late in its clinical course, usually with serious and/or life-threatening advanced symptoms such as heart failure, stroke from mural thrombus or sudden cardiac death[143].

Clinical characterization

Patients with DCM often present with signs and symptoms of pulmonary congestion, low cardiac output and fatigue[144]. The diagnosis of DCM is established during echocardiography by the presence of a left ventricular cavity dimension of > 112% of predicted normal values and a shortening fraction of < 25% defining abnormal systolic function[145]. The histological changes associated with DCM include myocyte death, interstitial fibrosis, and myocyte morphological alterations (Figure 8), which typically include nuclear hypertrophy and an empty appearance due to myofibril loss.

An additional form of DCM is the peripartum cardiomyopathy (PPCM) unique to pregnant women during the last month of pregnancy and up to six months postpartum[143]. The cause of PPCM is not fully understood, however the disease is suggested to be triggered by late-gestinal maternal hormones and genetic predispositions[146]. The incidence of PPCM is low in pregnant women (1:1000), but the mortality ranges from 7-50%[147].

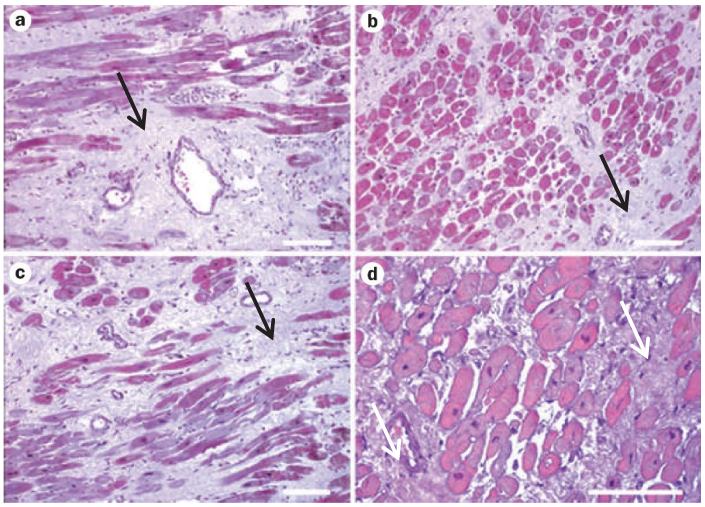


Figure 8 Typical pathological findings in DCM (copied from Roura *et al.*)[148].

Histopathological alterations in the myocardium of a patient with DCM stained with A-C) Masson's Trichrome and D) hematoxylin and eosin showing few blood vessels and marked collagen deposition (white arrows) and interstitial fibrosis (black arrows) surrounding myocardial filaments and vasculature. Increased myocyte atrophy and loss are also microscopic characteristics found in the myocardium of these patients. Scale bars, 50μm.

Genetic background

The primary mode of inheritance for idiopathic DCM is autosomal dominant and at least 25% of patients have evidence for familial disease[144]. Autosomal-recessive mutations are a less common cause of idiopathic DCM, although X-linked recessive inheritance is associated with several genes such as dystrophin (*DMD*), tafazzin (*TAZ*), and emerin (*EMD*).

Mutations reported to cause DCM have been described in more than 50 genes (Table 6), which are involved in a wide range of functions including the sarcomere, cytoskeleton, nuclear envelop, desmosomal complexes, calcium handling, and transcription factors (Figure 9)[149] .

Causative mutations in calcium regulating proteins and disturbed ion channel functions have been identified in the ryanodine receptor 2 *(RYR2*) and phospholamban (*PLN*) (Figure 9A)[150]. Ryanodine receptors are located in the sarcoplasmic/endoplasmic reticulum membrane and are responsible for the release of Ca^{2+} from intracellular stores during cardiac excitation-contraction coupling in cardiomyocytes[151]. Dysfunctional RyR2 receptors contribute to an increase in cytosolic calcium concentration, which can result in delayed after-depolarization triggering lethal arrhythmias[152]. Phospholamban is a regulator of calcium uptake into the sarcoplasmic reticulum via inhibition of Ca^{2+}-ATPase (*SERCA2*) in cardiomyocytes[153].

The sarcomere is the fundamental contractile unit of the cardiac muscle (Figure 9B and C)[154]. Each sarcomere is composed of parallel alternating thin actin filaments and thick myosin rods that are anchored to the Z-line[155]. Muscle contraction occurs when the central bipolar myosin thick filaments use ATPase-generated force to pull the thin filaments, sliding the two types of filaments across each other to reduce sarcomere length[156]. Mutations in the β-myosin heavy chain 7 encoding gene *MYH7*, the α-myosin heavy chain 6 encoding gene *MYH6*, the myopalladin encoding gene *MYPN,* and the titin encoding gene *TTN* account for 30% of cases with DCM and

are therefore the most common genes within the large heterogeneity of DCM [150]. Although it is known that disease-causal gene alterations in sarcomeric proteins show overlaps between DCM and HCM, the underlying mechanisms responsible for these differences are not yet understood[141]. The two isoforms of the nuclear proteins lamin A and C (*LMNA*) are intermediate filaments which form the lamina of the nuclear envelope (Figure 9E). LMNA mutations are not only associated with DCM, but with a variety of phenotypes including Limb-Girdle muscular dystrophy, Emery-Dreifuss muscular dystrophy and autosomal dominant partial lipodystrophy[157]. DCM patients with LMNA mutations show an early onset of disease, have cardiac conduction disturbances and skeletal muscle involvement with high creatinine kinase levels and are at high risk for life-threatening ventricular arrhythmias and sudden death[158]. Other proteins of the nuclear envelope that interact with Lamin A/C are thymopoietin (*TMPO*)[159] and emerin (*EMD*)[160].

Dystrophin-associated glycoprotein complexes and intermediate filament proteins are structural proteins that are important to stabilize organelles by linking the Z-line to the sarcolemma (Figure 9F)[161]. Desmin (*DES*) and dystrophin (*DMD*) form filamentous systems that link the sarcomere to sarcolemma and extracellular matrix[162].

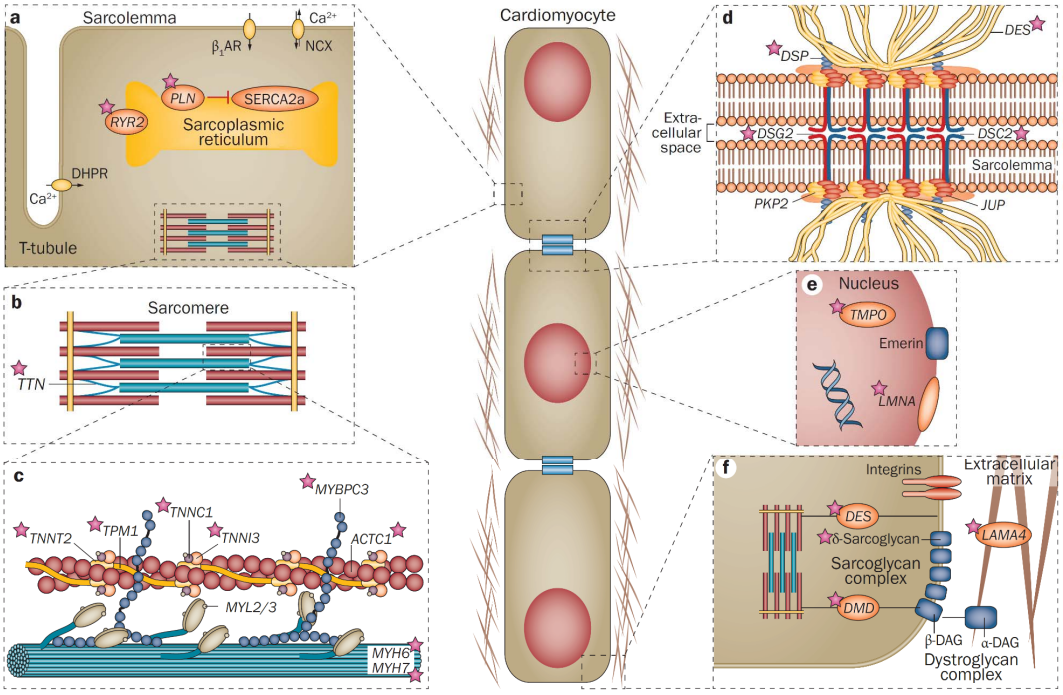


Figure 9 A remarkably heterogeneous set of rare genetic variants yield the phenotype of DCM (copied from Hershberger *et al.*)[141].

More than 50 genes have been implicated in DCM which are distributed across a variety of cellular ontologies and biochemical pathways. The majority of them can be directly associated with cardiac muscle contraction and regulation. These functions include a) sarcolemma protein encoding genes, b) and c) sarcomere protein encoding genes, d) desmosome protein encoding genes, e) nuclear envelope encoding genes, and f) cytoskeletal protein encoding genes. Genes in which a DCM-associated mutation has been identified are indicated by red star.

Gene	Protein name	Protein localisation / Function	Estimated prevalence
ABCC9	ATP-binding cassette, subfamily C, member 9	K^+-channel regulatory activity	<1%
ACTC1	Actin, α, cardiac muscle 1	Sarcomere, muscle contraction	<1%
ACTN2	Actinin, α2	Sarcomere, anchor for myofibrillar actin	1%
ANKRD1	Ankyrin repeat domain-containing protein 1	Sarcomere, localized to myopalladin	rare
BAG3	Bcl2-associated athanogene 3	Sarcomere, inhibits apoptosis	rare
CTF1	Cardiotrophin 1	Intra/extracellular-cytokine, messenger	rare
CRYAB	α-Crystallin B	Cytoskeletal protein, protein binding	rare
CSRP3	Muscle LIM protein	Sarcomere, stretch sensor	<1%
DES	Desmin	Cytoskeleton, transduces contractile forces	<1%
DMD	Dystrophin	Cytoskeleton, transduces contractile forces	rare
DNAJC19	DNAJ homolog, subfamily C, member 19	Heat shock protein family	rare
DSC2	Desmocollin-2	Desmosome, links desmosomes of neighbouring cells	NA
DSG2	Desmoglein-2	Desmosome, links desmosomes of neighbouring cells	NA
DSP	Desmoplakin	Desmosome, tethers intermediate filaments to desmosomal plaques	NA
EMD	Emerin	Nuclear membrane, protein binding	rare
EYA4	Eyes absent homolog 4 (Drosophila)	Nucleus, transcriptional coactivators	rare
FHL2	Four and a half LIM domains 2	Sarcomere, protein-binding	rare
FKTN	Fukuyama-type congenital muscular dystrophy	Nucleus	rare
GAA	Glucosidase, alpha acid	Lysosomes, enzymatic activity	rare
GATAD1	GATA zinc finger domain containing 1	Nucleus, histone modification	rare
ILK	Integrin-linked kinase	Cytoskeleton, interacts with integrin	<1%
LAMA4	Laminin, alpha 4	Cytoskeleton, extracellular matrix protein	1%
LDB3	LIM binding domain 3 (ZASP)	Cytoskeleton, clustering of membrane proteins	1%
LMNA	Lamin A/C	Nuclear envelope, stability of inner nuclear membrane, regulation of gene expression	**6%**
MYBPC3	Myosin-binding protein C	Sarcomere, muscle contraction	2%
MYH6	α-Myosin, heavy chain 6	Sarcomere, muscle contraction	**4%**
MYH7	β-Myosin, heavy chain 7	Sarcomere, muscle contraction	**10%**
MYPN	Myopalladin	Sarcomere, Z-disc protein	**3-4%**
NEBL	Nebulette	Sarcomere, protein binding	rare
NEXN	Nexilin (F actin-binding protein)	Sarcomere, protein binding	<1%
PDLIM3	PDZ LIM domain protein 3	Cytoskeleton, cytoskeleton protein	<1%
PLN	Phospholamban	Sarcoplasmic reticulum, protein binding	<1%
PSEN1	Presenilin-1	γ-secretase activity, transmembrane protein	<1%
PSEN2	Presenilin-2	γ-secretase activity, transmembrane protein	<1%

Table 6 Genes associated with DCM.

Gene	Protein name	Protein localisation / Function	Estimated prevalence
RBM20	RNA-binding motif protein 20	Spliceosomal, regulates splicing of several cardiac genes	2%
SCN5A	Sodium voltage-gated channel α-subunit type 5	Ion channel, controls Na^+ flux	2-3%
SDHA	Succinate dehydrogenase complex, Flavoprotein subunit A	Mitochondrial, enzymatic activity	rare
SGCD	Sarcoglycan delta	Cytoskeleton, transduces contractile forces	<1%
TAZ	Tafazzin	Mitochondrial, enzymatic activity	rare
TCAP	Titin cap	Sarcomere, sarcomere assembly	1%
TMPO	Thymopoietin	Nuclear envelope, lamin-associated nuclear protein	1%
TNNC1	Troponin C type 1	Sarcomere, muscle contraction	<1%
TNNT2	Troponin T type 2	Sarcomere, muscle contraction	3%
TNNI3	Troponin I type 3	Sarcomere, muscle contraction	<1%
TPM1	Tropomiosin α-1 chain	Sarcomere, muscle contraction	<1%
TTN	Titin	Sarcomere, extensible scaffold	**10-20%**
TTR	Transthyretin	Cytoplasm, transport protein	rare
TXNRD2	Thioredoxin reductase 2	Cytoplasm, enzymatic activity	rare
VCL	Metavinculin	Cytoskeleton, protein binding	rare
XK	x-linked Kx blood group	Membrane, protein binding	rare
ZASP	ZO-2 associated speckle protein	Sarcomere, protein binding	rare

Table 6 Genes associated with DCM.

Treatment

The primary aims of treatment are to control symptoms and to prevent disease progression and sudden cardiac death by pharmacologic therapy, pacemakers, or ICD[163]. Cardiac transplantation remains the definite treatment for advanced heart failure management in patients with intractable heart failure symptoms and end stage disease[164].

1.4.3. Hypertrophic cardiomyopathy (HCM)

Hypertrophic cardiomyopathy (HCM) is characterized by asymmetrical left ventricular remodeling with a small ventricular cavity dimension[165]. With a prevalence of 1:500, HCM is a relatively common cardiovascular disease in the general population and shows a variety of inter- and intrafamilial variations ranging from benign to malignant forms with a high risk of cardiac failure and sudden cardiac death[140]. HCM can present early in life with symptoms or patients can live for decades asymptomatically[166].

Clinical characteristics

HCM is defined by an abnormal left ventricular thickening without chamber dilatation in the absence of identifiable causes such as aortic valvular stenosis or hypertension (Figure 10A)[167]. The major underlying structural abnormalities in HCM are coronary microvasculature dysfunction by increased wall/lumen ratio, myocardial cell disarray and remodeling changes (Figure 10B)[166]. These abnormalities can lead to impaired coronary reserve, diastolic dysfunction, ventricular dysrhythmia, and sudden death. The most frequent symptoms of HCM include exercise intolerance, angina, dyspnea, dizziness, and syncope[168]. A diagnosis of HCM is made based on Doppler monitoring, magnetic resonance imaging, and computed tomography imaging including the assessments of ventricular dimensions, systolic and diastolic function, mitral valve function and geometry[169].

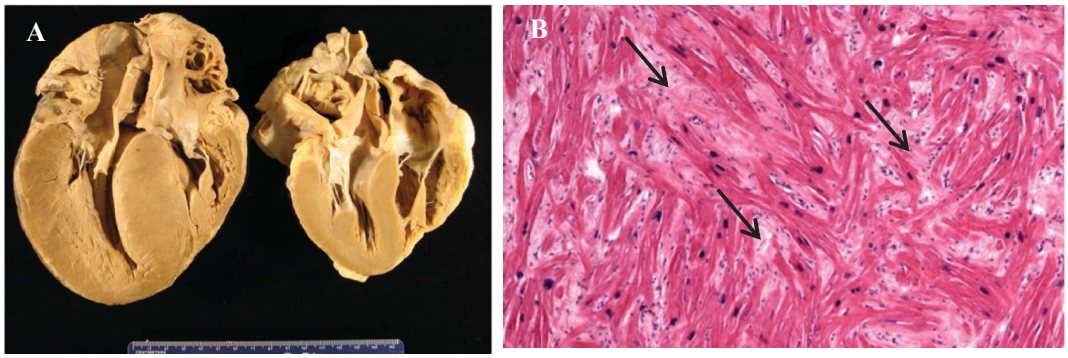


Figure 10 Pathological features of HCM (copied from Ho *et al.*)[170].

A) Gross pathology showing HCM (left) as compared to normal cardiac morphology (right). B) Histological sections stained with hematoxylin and eosin demonstrate myocyte disarray, where myocytes are oriented at bizarre and variable angles to each other, as well as increased myocardial fibrosis (black arrows).

Genetic background

HCM is inherited in an autosomal dominant pattern with a large genetic heterogeneity[171]. Several molecular and clinical genetic studies have linked HCM to mutations in genes encoding sarcomeric or sarcomere-associated proteins (Table 7)[172]. Approximately 70% of successfully genotyped patients are found to have mutations in *MYH7* and myosin-binding protein C gene (*MYBPC3*)[166]. Several other genes are less common and account only for < 5% of HCM patients, whereas the majority of these variants are found only in one family[173]. The overall mutation detection rate in HCM is very variable and ranges between 20 - 60%[174].

Gene	Protein name	Protein localisation / Function	Estimated prevalence
ACTC1	Actin, α, cardiac muscle 1	Sarcomere, muscle contraction	rare
ACTN2	Actinin, α2	Sarcomere, anchor for myofibrillar actin	rare
ANKRD1	Ankyrin repeat domain-containing protein 1	Sarcomere, localized to myopalladin	rare
CALR3	Calreticulin 3	Sarcoplasmic reticulum, protein binding	rare
CAV3	Caveolin 3	Sarcolemma, regulatory activity	rare
CSRP3	Muscle LIM protein	Sarcomere, stretch sensor	rare
JPH2	Junctophilin 2	Junctional complex, calcium handling	rare
LAMP2	Lysosome associate membrane glycoprotein 2	Cytoplasm, protein binding	rare
MYBPC3	Myosin-binding protein C	Sarcomere, muscle contraction	**40%**
MYH6	α-Myosin, heavy chain 6	Sarcomere, muscle contraction	rare
MYH7	β-Myosin, heavy chain 7	Sarcomere, muscle contraction	**40%**
MYL2	Myosin light chain 2	Sarcomere, protein binding	rare
MYL3	Myosin light chain 3	Sarcomere, protein binding	1%
MYLK2	Myosin light chain kinase 2	Sarcomere, kinase	rare
MYOZ2	Myozenin 2	Sarcomere, protein binding	rare
NEXN	Nexilin (F actin-binding protein)	Sarcomere, protein binding	rare
PRKAG2	Protein kinase, AMP-activated, 2 noncatalytic subunit	Cytosol, enzymatic activity	rare
TCAP	Titin cap	Sarcomere, sarcomere assembly	rare
TNNC1	Troponin C type 1	Sarcomere, muscle contraction	rare
TNNT2	Troponin T type 2	Sarcomere, muscle contraction	rare
TNNI3	Troponin I type 3	Sarcomere, muscle contraction	**5%**
TPM1	Tropomiosin α-1 chain	Sarcomere, muscle contraction	2%
TTN	Titin	Sarcomere, extensible scaffold	rare
VCL	Metavinculin	Cytoskeleton, protein binding	rare

Table 7 Genes associated with HCM.

Treatment

It is recommended that HCM patients should not participate in most competitive sports[175]. Treatment modalities include pharmacologic therapy, invasive septal reduction therapy, pacemakers, and ICD[176].

1.4.4. Left ventricular non-compaction cardiomyopathy (LVNC)

Left ventricular non-compaction (LVNC) is characterized by a soft and spongy myocardium of the left ventricle[177]. Whether LVNC is a distinct cardiomyopathy or a morphologic trait shared by

different cardiomyopathies remains still controversial[178]. The prevalence of LVNC is reported as one in 10'000 and men are more frequently affected than women[179]. The age of onset is highly variable with clinical expression varying from asymptomatic to progressively poor cardiac function, ventricular hypertrophy, increased thromboembolic events, and SCD[180].

Clinical characterization

The genesis of LVNC has been hypothesized to represent an arrest of myocardial compaction during the first trimester of embryonic development[177]. The gross pathological appearance of LVNC is characterized by excessively prominent trabeculations and deep intratrabecular recesses resembling the right ventricular endomyocardial morphology[181]. A variety of histological patterns have been noted including anastomosing broad trabeculae, coarse trabeculae, and sponge-like interlacing smaller muscle bundles[182]. LVNC can occur as an isolated myocardial trait or be associated with cardiomyopathies, congenital heart diseases, or complex syndromes affecting multiple organs and tissues, including metabolic derangements or neuromuscular disorders[183]. Although LVNC is usually asymptomatic, complications can manifest clinically as chest pain, dyspnea, palpitations, syncope, falls, leg edema, exercise intolerance, embolic ischemic stroke, myocardial infarction, or peripheral embolism[184].

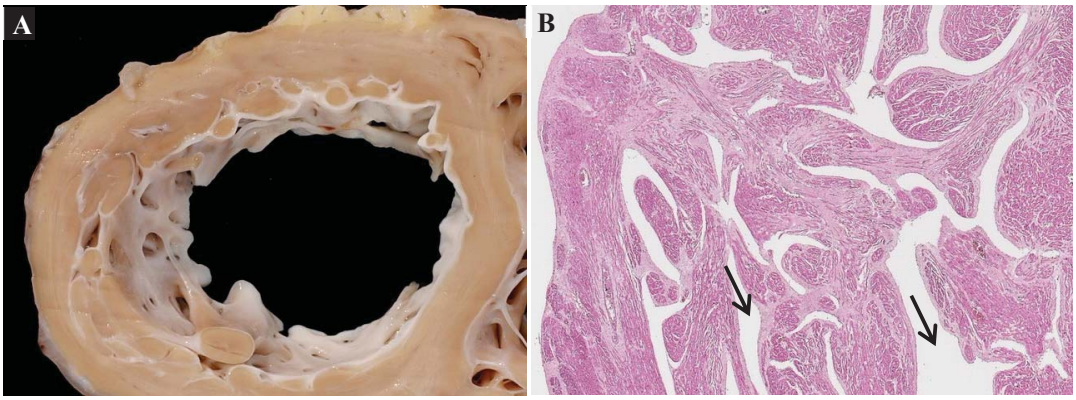


Figure 11 Pathological features of LVNC (copied from Ramnani *et al.*)[185].

A) LVNC presents with a diversified pattern, including anastomosing broad trabeculae and soft and spongy surface. B) Histological sections demonstrate deep intertrabecular recesses (black arrows) in the myocardium which communicate with the ventricular cavity (eosin-hematoxylin staining).

Genetic background

The inheritance pattern of LVNC is autosomal dominant or X-linked recessive and is often associated with other multisystem disorders. Although LVNC has been reported in association with mutations in more than 40 genes, a causal relationship between most of the variants and LVNC remains to be established[186].

The first mutation described in LVNC without evidence of congenital heart disease was identified in the X-linked tafazzin encoding gene *TAZ*[187]. Tafazzin is a phospholipid transacylase that is important in membrane function and mutations typically result in Barth syndrome, characterized by cardiomyopathy, skeletal myopathy, and prepubertal growth delay[188]. Several mutations causing autosomal dominant LVNC have been identified in sarcomere- and cytoskeleton-encoding genes such as *ACTC1*, *MYBPC3*, *MYH7*, *LMNA, TNNT2*, *TNNI3* and *TPM1* which account for 30% of LVNC[189]. Genetic testing has a detection rate of around 35-40%[190].

Gene	Protein name	Other associated diseases	Estimated prevalence
ACTC1	Actin, α, cardiac muscle 1	HCM	1%
DMD	Dystrophin	Dystrophinopathy	rare
DMPK	Dystrophia myotonica protein kinase	Myotonic dystrophy type 1	rare
DSP	Desmoplakin	ARVC, DCM	rare
DTNA	Dystrobrevin alpha	Dystrobrevinopathy	rare
FLNA	Flamin A	Melnick Fraser syndrome	rare
HCN4	Hyperpolarization-activated cyclic nucleotide–gated potassium channel 4	BrS	rare
LAMP2	Lysosome associate membrane glycoprotein 2	Danon disease, HCM	rare
LDB3	LIM binding domain 3 (ZASP)	Zaspopathy	rare
LMNA	Lamin A/C	Emery-Dreifuss muscular dystrophy	1%
MYBPC3	Myosin-binding protein C	DCM, HCM	**5-8%**
MYH7	β-Myosin, heavy chain 7	DCM, HCM	**13%**
NKX2-5	NK2 homeobox 5	Atrial septal defect, Tetralogy of Fallot	rare
PLN	Phospholamban	DCM, HCM	rare
TNNT2	Troponin T type 2	DCM, HCM	1%
TPM1	Tropomiosin α-1 chain	DCM, HCM	1%
TNNI3	Troponin I type 3	DCM, HCM	1%
RYR2	Ryanodine receptor 2	ARVC, CPVT	rare
SCN5A	Sodium voltage-gated channel α-subunit type 5	BrS, LQTS	rare
TAZ	Tafazzin	Barth syndrome	rare
VCL	Metavinculin	DCM, HCM	rare
ZASP	ZO-2 associated speckle protein	Barth syndrome, Zaspopathy	rare
ZNF9	Zinc finger protein 9	Myotonic dystrophy type 2	rare

Table 8 Genes associated with LVCN.

Treatment

Therapy for LVNC is largely dictated by concomitant clinical findings associated with myocardial dysfunction or significant arrhythmias. Patients with normal left ventricular size and function undergo clinical monitoring, whereas symptomatic patients with left ventricular dilation and dysfunction or hypertrophy may be clinically managed according to phenotype.

1.5. Cardiac channelopathies

The electrical activity of the human heart depends on the spontaneous generation and propagation of action potentials in specialized pacemaker cells in the sinoatrial node of the right atrium (Figure 12)[191]. The generated electrical impulse travels via the atrioventricular node along the bundle of His into the Purkinje fibers down to the ventricles, leading to ventricular contraction and ejection of the blood into the body. The different phases of cardiac electrical activity can be recorded by non-invasive surface electrocardiography (ECG).

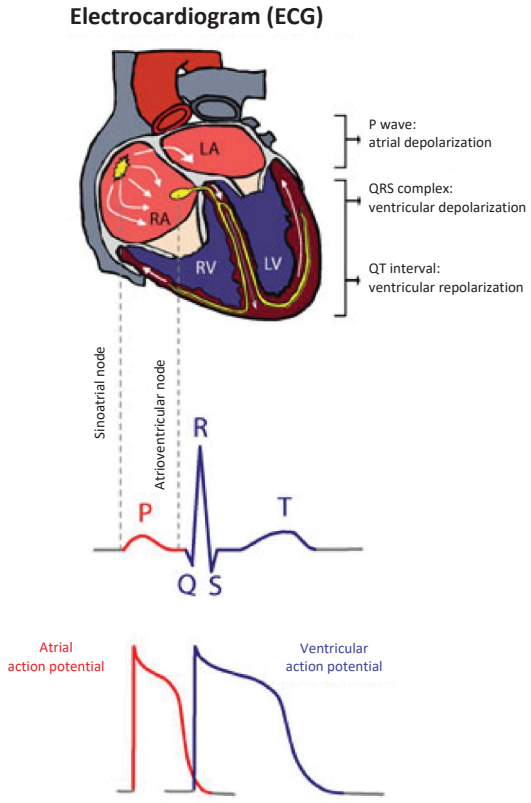


Figure 12 Cardiac electrical activity of the human heart (copied from Amin *et al.*)[192].

The electrical activity of the heart is represented by a 12-lead surface ECG, and results from coordinated action potential generation and propagation through individual cardiomyocytes. The P wave of the ECG records the action potential propagation between the sinoatrial node and the atrioventricular node and the QRST complex represents the action potential of the ventricles.

The overall magnitude of the electrical potential is measured by 12 different angles in the body over a period of time to capture the electrical activity at each moment throughout the cardiac cycle.

Myocardial action potentials are generated by sequential activation and inactivation of transmembrane ion channels that conduct depolarizing inward and repolarizing outward currents (Figure 13)[192]. The resting potential in cardiomyocytes is -90 mV due to constant outward leak of potassium (K^+) ions through inward rectifier channels. An action potential triggered in a neighboring cardiomyocyte or pacemaker cell causes the transmembrane potential to rise above -90 mV. During phase 0, depolarization is the result of an inward flux of positive charged sodium (Na^+) ions through fast opening Na^+-channels, which moves the negative resting membrane potential towards a more positive voltage value[193]. Partial fast repolarization is achieved by the rapid inactivation of Na^+-channels and an outward flux of positive charged K^+- ions in phase 1. The plateau in phase 2 is generated by an electrical balance between K^+-ion efflux and calcium (Ca^{2+}) ion influx causing a prolongation of the action potential duration by approximately 200-300 ms. The terminal repolarization during phase 3 is accomplished by slowly activating delayed rectifying K^+-channels that bring the membrane potential back to the resting potential.

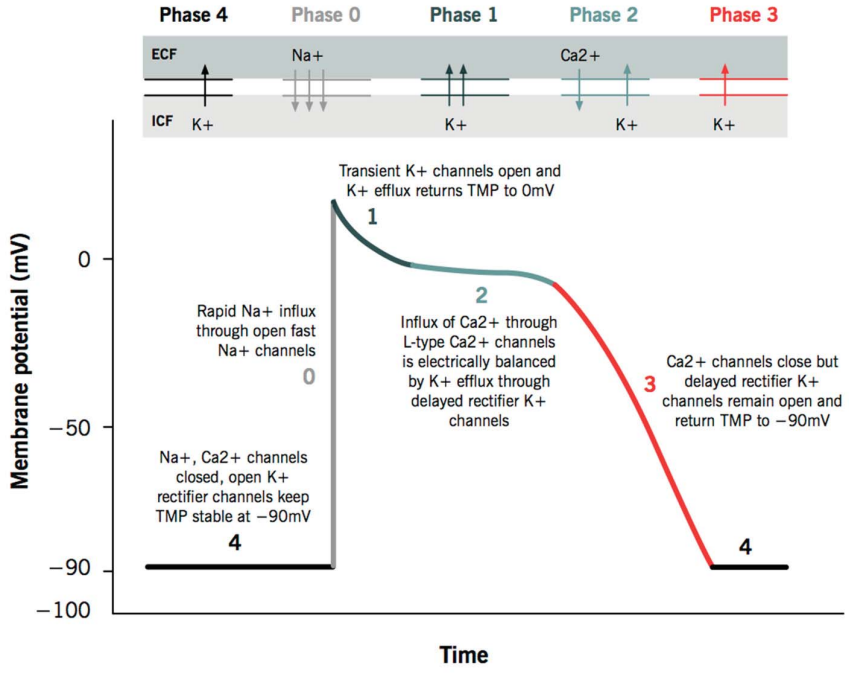


Figure 13 Cardiac action potential[194].

The ventricular cardiac action potential is generated by transmembrane inwardly and outwardly directed ion currents. The inward depolarizing sodium (Na^+) and calcium (Ca^{2+}) currents are illustrated by downwards pointing arrows (phase 0 and 2) and the outward repolarizing potassium (K^+) currents are illustrated by upwards pointing arrows (phase 4, 1, 2, and 3). ECF = extracellular flux, ICF = intracellular flux.

Voltage-gated ion channels are multimeric transmembrane proteins formed by pore-forming α-subunits, which mediate ion currents, and regulatory β-subunits[195]. The dysfunction of such ion channels, also referred to as channelopathies, can lead to changes in action potential waveforms, synchronization, and/or propagation, thereby predisposing the heart to potentially life-threatening arrhythmias[196]. The most common form of a cardiac channelopathy is the long QT syndrome (LQTS), followed by the Brugada syndrome (BrS), catecholaminergic polymorphic ventricular cardiomyopathy (CPVT), and short QT syndrome (SQTS) (Table 9)[197].

	BrS	CPVT	LQTS	SQTS
Clinical characterization	• Unique coved – type ST-segment elevation in the right precordial leads • Symptoms include agitation, and agonal respiration • Modulators are fever, drugs, and medications	• Episodic syncope during exercise or acute emotional stress • Symptoms include dizziness and palpitations	• QT interval prolongation (>470ms) • *torsade de pointes* (TdP)-mediated syncope • Symptoms include dizziness, syncope, and cardiac arrest	• QT interval shortening (<330ms) • Atrial and ventricular tachyarrhythmia
Prevalence	• 1:2'000	• 1:10'000	• 1:2'000	• 1:10'000
Age of onset	• Around 40 years of age	• Between 7 and 12 years of age	• Adolescents and young adults	• Children and adults
Genetics	• 24 genes • Major gene is *SCN5A*	• 4 genes • Major genes are *CASQ2* and *RYR2*	• 15 genes • Major genes are *KCNH2*, *KCNQ1*, and *SCN5A*	• 6 genes • Major genes are *KCNH2* and *KCNQ1*
Mutation detection rate*	• 30-35%	• 50-60%	• 60-75%	• 15-25%
Therapy and surveillance	• Avoidance of high fever, alcohol, anesthetics, antidepressant drugs, and antipsychotic drugs with sodium-blocking effect • ECG monitoring every one to two years • ICD	• Avoidance of competitive sports and stress-related exercise • β-blockers • Flecainide • ICD • Follow-up every six to twelve months	• Avoidance of competitive sports, and stress-related emotions • β-blockers • ICD	• ICD

Table 9 Classification of cardiac channelopathies.

Br = Brugada syndrome, CPVT = catecholaminergic polymorphic ventricular tachycardia, LQTS = long QT syndrome, SQTS = short QT syndrome, ICD = implantable cardioverter defibrillator. * = percentage of detected variants in genetic screening.

1.5.1. Brugada syndrome (BrS)

Brugada syndrome (BrS) is described by the presence of right bundle branch block and persistent ST-segment elevation in the right precordial leads in a structurally normal heart[198]. The prevalence of BrS is estimated to be at three to five in 10'000 individuals of the general population, however an ethnicity-dependent occurrence has been reported with a higher frequency in the Southeast Asian population[199]. The clinical phenotype manifests in adulthood around 40 years of age and is 8 to 10-times more frequent in males than in females[200]. The strong gender disequilibrium ratio is likely due to the influence of hormones and gender-dependent distribution of ion channels across the heart[201]. Currently, BrS is suggested to be responsible for 4% of all SCD cases in the general population and up to 12% of sudden death in patients with structurally normal hearts[202].

Clinical characterization

The clinical diagnosis of BrS requires the identification of unique coved-type ST-segment elevation in the right precordial leads at baseline or after the use of sodium blockers such as ajmaline, flecainide, or procainamide (Figure 14)[203].

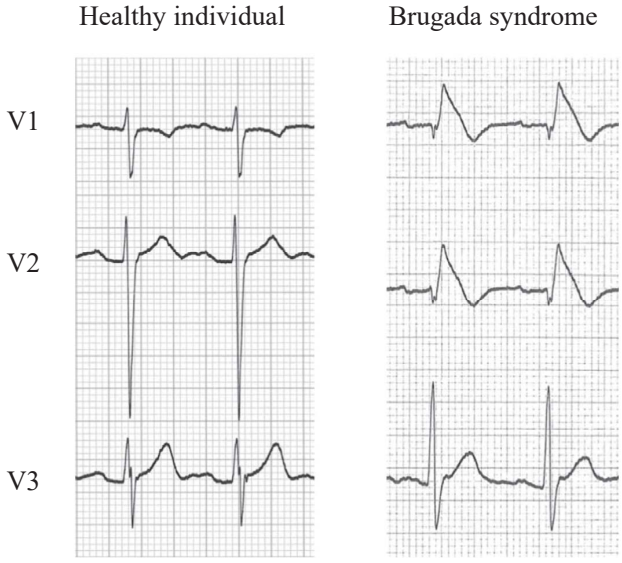


Figure 14 Electrocardiogram of leads V1-V3 showing typical cove-typed ST-segment elevation in lead V1 and V2 in a patient with Brugada syndrome (right) compared to a healthy individual (left) (copied from Watanabe *et al.*)[204].

The first manifestation of the disease usually occurs during rest or sleep likely due to high vagal tone[205]. Additional symptoms include seizure, agitation, agonal respiration, and not uncommonly recent memory loss. Several environmental modulators play a major role in the dynamic nature of the ECG and may also be responsible for ST-segment elevation[201]. The most well-known environmental risk factor is a high body temperature as fever has been reported not only to

accentuate the BrS ECG pattern but also to temporary increase the risk of arrhythmias[206]. Another risk factor is a cocaine overdose which has a potent sodium channel blocking effect resembling that of flecainide[207]. Similarly, several medications, anti-depressants, anti-arrhythmic and anesthetics have been associated with an increased risk of ST-elevation and arrhythmias in genetically predisposed individuals[208].

Genetic background

BrS exhibits an autosomal dominant pattern of inheritance and a family history of unexplained sudden death is present in 20-30% of patients with BrS. More than 450 pathogenic variants associated with BrS have been reported in 24 different genes, primarily encoding sodium, potassium, and calcium channels, or proteins associated with these channels[209]. Despite the large number of BrS-susceptibility genes, only about 30-35% of BrS cases can genetically be diagnosed, and most of them (25-30%) result from pathogenic variants in the sodium channel $Na_v1.5$ α-subunit encoding gene *SCN5A*[204]. $Na_v1.5$ predominates the cardiac sodium current (I_{Na}), which underlies the fast upstroke of the cardiac action potential. Due to the important function of $Na_v1.5$, *SCN5A* is described as a candidate gene in multiple cardiac diseases including, BrS, long QT syndrome, conduction disease, and cardiomyopathies[210]. Additional variants have been identified in the regulatory β-subunit encoding genes *SCN1B-SCN3B*[89, 211, 212]. Apart from sodium channels, several potassium channels have been related to BrS, including the potassium voltage-gated channel, Isk-related family, member 3 gene *KCNE3*[213], potassium inwardly rectifying channel, subfamily J, member 8 gene *KCNJ8*[214], potassium voltage-gated channel, Shal-related subfamily, member 3 gene *KCND3*[79], and potassium voltage-gated channel, Isk-related family, member 5 gene *KCNE5*[215]. However, variants in these genes only account for about 5% of all BrS cases.

Gene	Protein Name	Function	Estimated prevalence
ABCC9	ATP binding cassette, subfamily C, member 9	K^+-channel regulatory activity	<1%
CACNB2	Calcium voltage-gated channel, subunit β2	Ion channel activity	**1-3%**
CACNA1C	Calcium voltage-gated channel, subunit α1C	Ion channel activity	**1-3%**
CACNA2D1	Calcium voltage-gated channel, subunit α2δ4	Ion channel activity	<1%
FGF12	Fibroblast growth factor 12	Na^+-channel regulatory activity	<1%
GPD1L	Glycerol-3-phosphate dehydrogenase 1-like	Na^+-channel regulatory activity	<1%
HCN2	Hyperpolarization-activated cyclic nucleotide-gated potassium channel 2	Ion channel activity	<1%
HCN4	Hyperpolarization-activated cyclic nucleotide–gated potassium channel 4	Ion channel activity	<1%

Table 10 Genes associated with BrS.

Gene	Protein Name	Function	Estimated prevalence
KCND3	Potassium voltage-gated channel, Shal-related family, member 3	Ion channel activity	<1%
KCNE3	Potassium voltage-gated channel, Isk-related family, member 3	Ion channel activity	<1%
KCNE5	Potassium voltage-gated channel, subfamily E, regulatory subunit 5	Ion channel activity	<1%
KCNH2	Potassium voltage-gated channel, subfamily H, member 2	Ion channel activity	<1%
KCNJ8	Potassium inwardly rectifying channel, subfamily J, member 8	Ion channel activity	<1%
PKP2	Plakophilin 2	Na^+-channel regulatory activity	<1%
RANGRF	RAN guanine nucleotide release factor	Na^+-channel regulatory activity	<1%
SCN5A	Sodium voltage-gated channel α-subunit type 5	Ion channel activity	**15-30%**
SCN10A	Sodium voltage-gated channel α-subunit type 10	Ion channel activity	<1%
SCN1B	Sodium voltage-gated channel β-subunit type 1	Ion channel activity	<1%
SCN2B	Sodium voltage-gated channel β-subunit type 2	Ion channel activity	<1%
SCN3B	Sodium voltage-gated channel β-subunit type 3	Ion channel activity	<1%
SCN4B	Sodium voltage-gated channel β-subunit type 4	Ion channel activity	<1%
SEMA3A	Semaphorin 3A	Semaphoring receptor binding	<1%
SLMAP	Sarcolemma associated protein	Protein binding	<1%
TRPM4	Transient receptor potential cation channel, subfamily M, member 4	Ion channel activity	<1%

Table 10 Genes associated with BrS.

Treatment

Treatment includes pharmacologic therapy, especially quinidine and phosphodiesterase III inhibitors, but the only proven effective strategy for preventing SCD in BrS patients is the use of ICD[216, 217].

1.5.2. Catecholaminergic polymorphic ventricular tachycardia (CPVT)

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is characterized by episodic syncope triggered by physical exercise or strong emotional stress[218]. The prevalence of CPVT is unknown in the general population, but has been estimated at one in 10'000[219]. First symptoms of the disease usually begin early in childhood between seven and twelve years of age, however atypical cases with start of the symptoms in the 3rd or 4th decade of life has been reported as well[220]. Even though it is a very rare disease, the mortality of CPVT is extremely high, reaching up to 30% by the age of 30 years in untreated individuals[218]. Therefore, CPVT plays an important role in SCD in the young and undiagnosed life-threatening CPVT is thought to contribute to the pathological process of sudden death in 10% of SUD and 1.5% of SIDS cases[221, 222].

Clinical characteristics

CPVT is one of the most malignant and yet insufficiently recognized primary electrical diseases of the heart[223]. The basal 12-lead ECG of patients with CPVT tends to be normal (Figure 15A), although 20% of the patients have been reported with sinus bradycardia[220]. Syncope is the first clinical manifestation of CPVT patients and less prevalent signs and symptoms include dizziness or palpitations[224]. During exercise testing, isolated and often monomorphic ventricular premature beats typically occur first, followed by polymorphic doublets or ventricular arrhythmias (Figure 15B), which disappear when the exercise is stopped[225]. Due to the occurrence of several generalized seizures, patients can be misdiagnosed with epilepsy and are treated with antiepileptic drugs prior to the elucidation of their cardiac channelopathy[226].

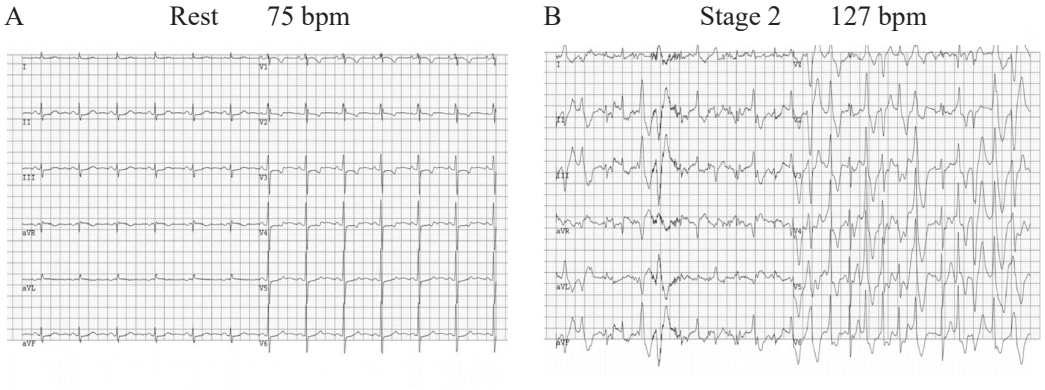


Figure 15 Polymorphic ventricular arrhythmias during a treadmill exercise test in patient with CPVT (adapted from Van der Werf *et al.*)[219].

A) ECG recording of a CPVT patient at rest. B) ECG during exercise, showing an increasing polymorphic ventricular arrhythmia burden, starting with isolated and bigeminal ventricular premature beats, and ending with bidirectional and polymorphic couplets and non-sustained ventricular tachycardia. bpm = beats per minute.

Genetic background

The familial occurrence of CPVT was already recognized in 1960 when three sisters presented with multifocal ventricular extrasystole during exercise or emotional stress in the absence of structural heart diseases[227]. Decades later, genetic linkage studies revealed a disease-causing locus with an autosomal dominant inheritance pattern on chromosome 1q42-q43[228]. Later, the *RYR2*-encoding ryanodine receptor/calcium release channel gene was identified at this locus and mutations in this gene have been reported in approximately 65% of CPVT patients[229].

An autosomal recessive inheritance pattern of CPVT was linked to mutations in the cardiac calsequestrin encoding gene *CASQ2* in a large Bedouin family[230]. Calsequestrin is a calcium buffering protein that contributes to the mobilization of Ca^{2+}-ions during each contraction in cardiac muscles[231]. Although mutations in *CASQ2* only account for 2-5% of the CPVT cases, they

cause a higher rate of SD than that observed with *RYR2* mutations[230]. Additional mutations in CPVT cases have been linked to *CALM1* and *TRDN*[232, 233].

Gene	Protein Name	Function	Estimated prevalence
CALM1	Calmodulin 1 (Phosphorylase kinase, delta)	Ca^{2+}-binding	<1%
CASQ2	Calsequestrin	Ca^{2+}-binding	2-5%
RYR2	Ryanodine receptor 2	Ion channel activity	**50-55%**
TRDN	Triadin	Ion channel binding	rare

Table 11 Genes associated with CPVT.

Treatment

Because of the poor prognosis of untreated CPVT, drug therapy is indicated for all clinically diagnosed patients and usually also for all silent carriers of *RYR2* mutations. First-line therapy for CPVT consists of β-blockers at the highest tolerable dose and it is recommend to avoid exhausting exercise and competitive sports[205]. When β-blockers fail to sufficiently suppress ventricular arrhythmias during exercise, the next therapeutic step are flecainide which has apart from the known $Na_v1.5$ blocking effect, a direct blocking effect on RyR2 channels. For CPVT patients, who experience cardiac arrest, recurrent syncope or ventricular tachycardia despite optimal medical therapy, ICDs are strongly recommended[205].

1.5.3. Long QT syndrome (LQTS)

Long QT syndrome (LQTS) is characterized by QT interval prolongation and increased risk for torsades des pointes (TdP)-mediated syncope, seizures and sudden cardiac death in the setting of a structurally normal heart[234]. The prevalence of LQTS has been assumed to range between one to four in 5'000 individuals[235]. More recent studies however suggested an even higher prevalence of at least one in 2'000 individuals due to the clinical heterogeneity varying from lifelong asymptomatic state to sudden death during infancy[236]. Moreover, gender is a major factor in determining the course and clinical manifestation of LQTS. Even though the QT interval duration is similar between young boys and girls, males are typically at higher risk during puberty and females from teenage life onward[237]. Changes in the QT duration are associated with sex hormone levels, menstrual cycle, pregnancy, and postpartum period[238]. Among symptomatic LQTS patients, the untreated 10-year mortality is approximately 50%[239].

Clinical characteristics

LQTS is identified by a prolonged QT interval on the surface 12-lead ECG (Figure 16), often after typical complaints such as dizziness, syncope, or cardiac arrest[240]. A normal duration of the heart-rate corrected QT (QTc) interval at rest is considered to be within 350-440 ms in healthy adults[193]. The diagnosis of LQTS is likely when cardiac symptoms accompany a markedly prolonged QT interval of more than 470 ms in adult males and 480 ms in adult females in the absence of another cause for QT prolongation such as medication or electrolyte disturbance[240]. Concealed LQTS may be unmasked by either resting ECG, exercise testing, or epinephrine challenge, which may lead to QT prolongation and T-wave morphological abnormalities.

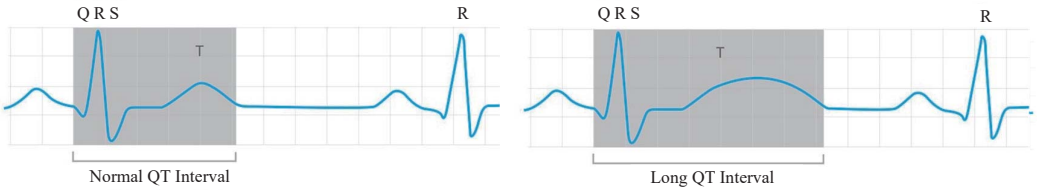


Figure 16 ECG measure of a healthy person (left) and a patient with prolonged QT interval (right) (adapted from http://patients.ambrygen.com/cardiology/about-the-diseases/long-qt-syndrome/diagnosis)[241].

The QT interval is defined from the beginning of the QRS complex to the end of the T wave. The normal QT interval duration is between 350 and 440 ms, whereas LQTS presents with a prolonged QT interval of more than 470 ms in adult males and 480 ms in adult females.

Genetic background

LQTS is typically inherited in an autosomal dominant manner. An exception is the Jervell and Lange-Nielsen syndrome, which is associated with sensorineural deafness and is inherited in an autosomal recessive manner[242]. LQTS displays great genetic heterogeneity with more than 500 pathogenic variants identified in at least 19 genes. The three major LQTS-susceptibility genes are *KCNQ1*, *KCNH2*, and *SCN5A* and mutations in these genes account for 75% of all positively genotyped patients. *KCNQ1* encodes the α-subunit of the voltage-gated potassium channel and mediates the slow component of the delayed rectifier potassium current (I_{Ks}). Pathogenic variants in *KCN1Q* cause a prolongation of the repolarization during phase 2 leading to an increased duration of the QTc interval[243]. *KCNH2* encodes the slow activated α-subunit of the voltage-gated potassium channel and mutations cause a reduction of I_{Kr} current, through mechanisms similar to the effects exhibited by KCNQ1 mutations on I_{Ks} current[244]. Gain-of-function variants in *SCN5A* induce an increased late inward $Na_v1.5$ current preventing cardiac repolarization, therefore also involved in the QT interval prolongation[245]. The remaining genes are responsible for nearly 10% of LQTS patients. The autosomal recessive Jervell and Lange-Nielsen disease has been associated

with homozygous or compound heterozygous mutations in the two genes *KCNQ1* and *KCNE1* that account for at least 80% of cases[246].

Gene	Protein Name	Function	Estimated prevalence
AKAP9	A-kinase anchoring protein 9	K^+-channel regulatory activity	<1%
ANK2	Ankyrin 2, neuronal	Protein binding	<1%
CACNB2	Calcium voltage-gated channel, subunit β2	Ion channel activity	<1%
CACNA1C	Calcium voltage-gated channel, subunit α1C	Ion channel activity	<1%
CACNA2D1	Calcium voltage-gated channel, subunit α2δ4	Ion channel activity	<1%
CALM1	Calmodulin 1 (Phosphorylase kinase, delta)	Ca^{2+}-binding	<1%
CALM2	Calmodulin 2 (Phosphorylase kinase, delta)	Ca^{2+}-binding	<1%
CAV3	Caveolin 3	Ion channel regulatory activity	<1%
KCNE1	Potassium voltage-gated channel, subfamily E, regulatory subunit 1	Ion channel activity	<1%
KCNE2	Potassium voltage-gated channel, subfamily E, regulatory subunit 2	Ion channel activity	<1%
KCNH2	Potassium voltage-gated channel, subfamily H, member 2	Ion channel activity	**25-30%**
KCNJ2	Potassium voltage-gated channel, subfamily J, member 2	Ion channel activity	<1%
KCNJ5	Potassium voltage-gated channel, subfamily J, member 5	Ion channel activity	rare
KCNQ1	Potassium voltage-gated channel, subfamily Q, member 1	Ion channel activity	**30-35%**
SCN5A	Sodium voltage-gated channel α-subunit type 5	Ion channel activity	5-10%
SCN4B	Sodium voltage-gated channel β-subunit type 4	Ion channel activity	rare
SNTA1	Syntrophin α1	Protein binding	rare

Table 12 Genes associated with SQTS.

Treatment

β-blockers are firmly established as first line therapy in LQTS and show a remarkable efficacy in the suppression of cardiac events[243]. Lifestyle advice is of particular importance in the management of LQTS, including the avoidance of QT-prolonging drugs, high-intensity exercises, and loud noise sources. Given the potential for sudden cardiac death, ICDs are frequently used in the management of LQTS, although the most of patients will be adequately protected by medical therapy with β-blockers.

1.5.4. Short QT syndrome (SQTS)

Short QT syndrome (SQTS) is characterized by abnormally short QT intervals and an increased propensity to develop atrial and ventricular tachyarrhythmia in the absence of a structural heart disease[247]. Currently, SQTS has been described in only a few families worldwide and the overall prevalence is estimated to be less than one in 10'000 individuals[248]. A peak of incidence is

observed during the first year of life, followed by a quiescent phase encompassing adolescence and another peak at older age[249].

Clinical characterization

The 12-lead surface ECG of SQTS patients is characterized by a strikingly short QTc interval typically below 360 ms in males and below 370 ms in females, virtual absence of the ST- segment, and tall, peaked, narrow-based T waves (Figure 17)[250]. Cardiac arrest seems to be the most frequent symptom, followed by palpitations, syncope, and atrial fibrillation[251]. Episodes of short QTc interval may occur in a wide range of situations such as a reaction to loud noise, at rest, during exercise, and during daily activity[252].

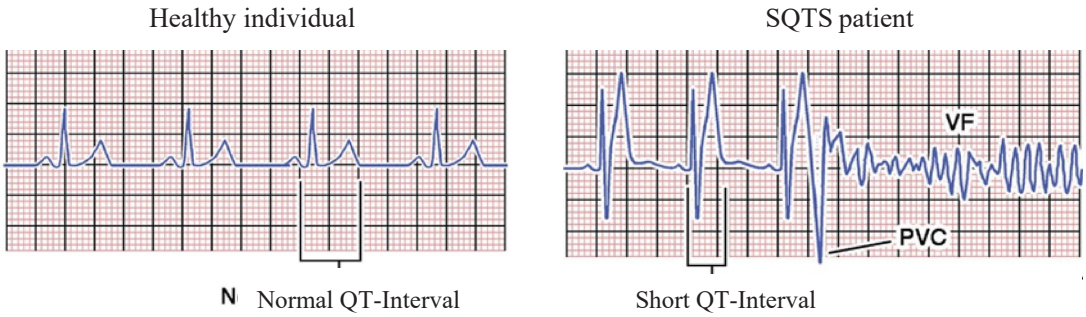


Figure 17 ECG measure of a healthy person and a patient with shortened QT interval (adapted from Gollob *et al.*)[253]. The main ECG abnormalities in SQTS are a short QT interval, peaked T waves, and short or absent ST segments. PVC = premature ventricular contraction, VF = ventricular fibrillation.

Genetic background

SQTS is inherited in an autosomal-dominant pattern and demonstrates a high penetrance. Sequence alterations in six genes have been associated with SQTS so far (Table 13). Despite the fact that a familial association is present in the majority of patients, the yield of genetic screening is low and varies between 15-25%[253]. Gain-of-function mutations of potassium and loss-of-function mutations of calcium channels result in an abbreviated repolarization phase during action potential and shortening of the QT interval. The most prevalent subtype of SQTS is associated with two different gain-of function variants in the potassium voltage-gate channel encoding gene *KCNH2*[254]. Another gain-of-function variant was identified in *KCNQ1* in a 70-year-old patient with abnormally short QTc duration of 300 ms and aborted SCD[255].

Gene	Protein Name	Function	Estimated prevalence
CACNB2	Calcium voltage-gated channel, subunit β2	Ion channel activity	< 1%
CACNA1C	Calcium voltage-gated channel, subunit α1C	Ion channel activity	< 1%
CACNA2D1	Calcium voltage-gated channel, subunit α2δ4	Ion channel activity	< 1%

Table 13 Genes associated with SQTS.

Gene	Protein Name	Function	Estimated prevalence
KCNH2	Potassium voltage-gated channel, subfamily H, member 2	Ion channel activity	< 1%
KCNJ2	Potassium voltage-gated channel, subfamily J, member 2	Ion channel activity	< 1%
KCNQ1	Potassium voltage-gated channel, subfamily Q, member 1	Ion channel activity	< 1%

Table 13 Genes associated with SQTS.

Treatment

Pharmacological therapy of patients with SQTS is still poorly defined, mainly due to the lack of large-scale studies. There is however some evidence that quinidine maybe useful in SQTS patients. Due to the malignant nature of SQTS, ICD is recommended in symptomatic SQTS patients who are either survivors of sudden cardiac arrest and/or have documented spontaneous sustained ventricular tachyarrhythmia with or without syncope[205]. A unique problem with ICDs in SQTS is that sometimes the tall and peaked T wave is misinterpreted as short interval, provoking an inappropriate shock from the ICD[256].

1.6. Post-mortem molecular autopsy in SIDS and SUD cases

In the past, large genetic screening studies in SIDS and SUD cases were difficult and time-consuming due to the limitations of sequencing strategies. In 2001, a first population-based "molecular autopsy" study in a large SIDS cohort investigated the protein-coding exons of *SCNA5,* which has emerged as the leading candidate ion channel gene for SIDS and identified *SCN5A* channel defects in approximately 2% of the cases[97]. Another post-mortem analysis of 12 adult SUD cases described a detection rate of 16.6% within the major five LQTS-associated genes (*KCNE1*, *KCNE2*, *KCNH2*, *KCNQ1*, and *SCN5A)*[257]. The inclusion of the CPVT-associated gene *RYR2* into the post-mortem molecular screening of 49 SUD cases identified possibly disease-causing variants in *RYR2* in 14% of the cases beside the 20% of cases which had variants within the five LQTS-associated genes[258, 259]. In the following years, several genetic studies in SIDS and SUD cases had focused on the identification of death-causing variants within the most promising candidate genes associated with cardiac diseases[222, 260-264]. Based on these genetic studies, it has been collectively suggested that up to 30% of SIDS/SUD cases might be explained by life-threatening arrhythmias in a morphological normal heart, therefore not detectable during conventional forensic autopsy investigations[5].

1.6.1. Post-mortem molecular autopsy based on whole-exome sequencing

Recently, enormous technical progress has been achieved in the areas of genotyping-array technologies and DNA sequencing[265]. Next-generation sequencing (NGS) approaches, also named as massive parallel sequencing (MPS), provide a comprehensive and time-efficient sequencing strategy to identify rare DNA sequence variants in the genome/exome of patients with complex disorders or to discover underlying genetic causes in large heterogeneous study populations[266]. Compared to traditional Sanger sequencing, MPS enables an increased detection rate of pathogenic variants, since a larger number of genes can be investigated and novel genes can be identified that may either cause or are associated with a particular disease[267].

Whole-exome sequencing (WES) is defined as the selective sequencing of all exons in a genome. In the human genome, the protein coding region constitutes about 1-2% of the whole genome, but it accounts for over 85% of all mutations that have been identified in Mendelian disorders[268, 269]. Today, WES or targeted sequencing of candidate genes are the most commonly used sequencing methods in disease diagnostics. Major advantages are a high throughput, well established sample preparation methods, and relatively low preparation and sequencing costs[270]. Additionally, sample preparation and sequencing can be performed from a small input amount of DNA, which can be crucial for post-mortem samples where the amount of available tissue and the DNA quality are limited.

In 2013, a first proof-of-principle case report of a WES-based molecular investigation identified a pathogenic *MYH7* mutation in an otherwise healthy 16-year-olf SUD victim, demonstrating that post-mortem molecular autopsy can increase the diagnostic yield[271]. Furthermore, post-mortem genetic analysis of SIDS/SUD victims may not only enable an accurate determination of the cause of death, but may also have significant impact for affected living first-degree family members in whom death-predisposing disorders had remained unrecognized so far[272].

1.6.2. The basic principles of whole-exome sequencing (WES)

DNA library preparation

The objective of NGS sample preparation is the clonal amplification of specific regions of the DNA[270]. First of all, genomic DNA is randomly fragmented by mechanical or enzymatic methods to a target size which depends on the platform read length and chemistry used. These DNA fragments are then used to generate a library by ligating universal DNA adaptors to both ends of the fragments, allowing complex genomes to be amplified with common PCR primers. After a pre-capture PCR amplification step, the library is enriched for sequences corresponding to the coding part of the genome. Biotinylated oligonucleotides (DNA or RNA baits) are hybridized to

the region of interest and magnetic biotin-streptavidin beads capture the biotinylated probes. The non-targeted portion of the genome is washed away, and samples are enriched by a post-capture PCR amplification step. The addition of a unique DNA sequence (barcode) to each fragment allows sample multiplexing and can potentially be introduced during the initial library construction or during post-capture amplification. In general, key performance parameters include the degree of enrichment, the uniformity with which targets are captured and the molecular complexity of the enriched library[273].

Clonal amplification

After library preparation, the samples are clonally amplified prior to the sequencing run. This step is crucial as most imaging systems have not been designed to detect single fluorescent or light signals. The most common methods are the emulsion PCR (emPCR) and solid-phase amplification (Figure 18). EmPCR is used to prepare sequencing templates in a cell-free system, which has the advantage of avoiding the arbitrary loss of genomic sequences[274]. Each DNA fragment is isolated in independent aqueous microreactors surrounded by an oil phase, and templates are amplified on primer-coated beads. Solid-phase amplification produces randomly distributed clonally amplified clusters of fragments on a glass slide[275]. High-density forward and reverse primers are covalently attached to the slide. The beads from the emulsion PCR are placed in picoliter-sized wells with one bead per well, whereas the slide from the bridge PCR is used directly for sequencing.

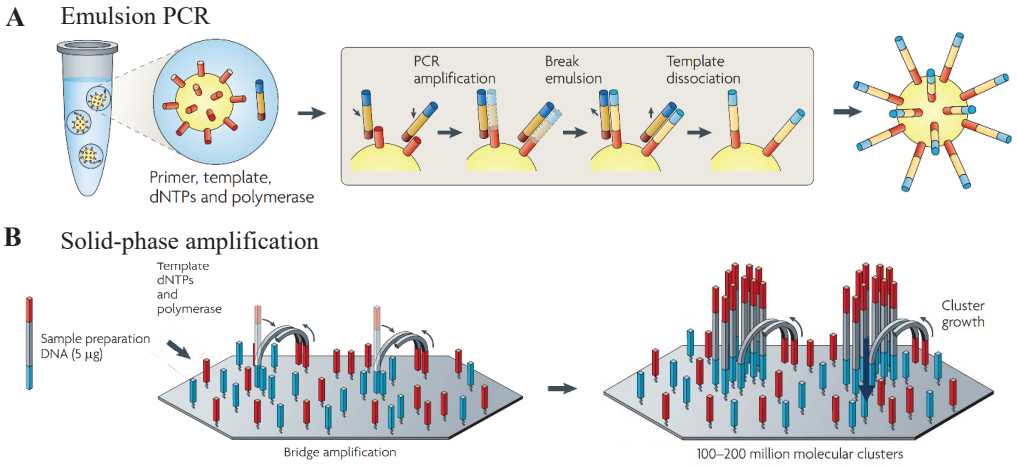


Figure 18 Template immobilization strategies (adapted from Metzker *et al.*)[276].

A) In emulsion PCR (emPCR), a reaction mixture consisting of an oil-aqueous emulsion is created to encapsulate bead-DNA complexes into single aqueous droplets. PCR amplification is performed within these droplets to create beads containing several thousand copies of the same template sequence. B) Solid-phase amplification is composed of two basic steps: initial priming and extending of the single-stranded, single-molecule template, and bridge amplification of the immobilized template with immediately adjacent primers to form clusters.

Sequencing platforms

The DNA sequence of each cluster is analyzed in real-time in a platform-specific procedure. In pyrosequencing (Roche 454 sequencing) and semi-conductor sequencing (Ion Torrent™), the nucleotides are added sequentially to the reaction (Figure 19). In some clusters, no DNA synthesis will take place because the added nucleotide cannot extend the growing strand. In others, one or more nucleotides will be added. The generated light signal in the pyrosequencing will be detected by a high-resolution charge-coupled device camera, whereas the pH-alteration of the semi-conductor sequencing will be detected on the sensor plate.

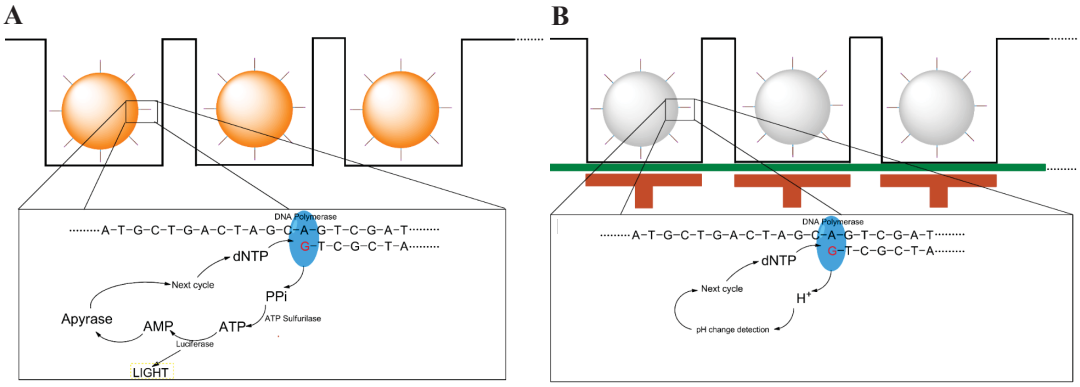


Figure 19 Pyrosequencing and semi-conductor sequencing steps (copied from Morey *et al.*)[270].

A) In the pyrosequencing process, a known dNTP is added to the reaction and incorporated by a polymerase to the complementary 3' positions adjacent to a universal sequencing primer. This generates one free pyrophosphate per base added. ATP sulfurilase will generate ATP by combining this pyrophosphate with adenosine 5' phosphosulfate. This ATP will be the luciferase substrate to generate visible light. After light measurement over each bead, the enzyme apyrase will degrade the non-incorporated nucleotides and a new sequencing cycle will begin. B) The sequencing reactions for the semi-conductor sequencing are performed in a microwell above a sensor plate. A known dNTP is added to the reaction and attached to the 3' end of a sequencing primer by a polymerase. By this, a hydrogen ion is released with each base added. The pH-alteration will be detected by the sensor plate. After the detection and washing, a new dNTP can be added to the reaction, repeating the process.

In the sequencing-by-synthesis method (lllumina®), all four fluorescently labeled nucleotides are present in the reaction, and one nucleotide is added to the growing DNA strand in all clusters. The nucleotides are reversibly blocked in the 3' position, which prevents incorporation of more than one nucleotide at the time. Imaging is then performed to record the cluster-specific fluorescence of the incorporated nucleotide. This is followed by a cleavage step, which removes the fluorescent dye, allowing the incorporation of the next nucleotide into the growing strand. The glass-slide of the Illumina HiSeq platform is partitioned into eight channels, which allows independent samples to be run simultaneously.

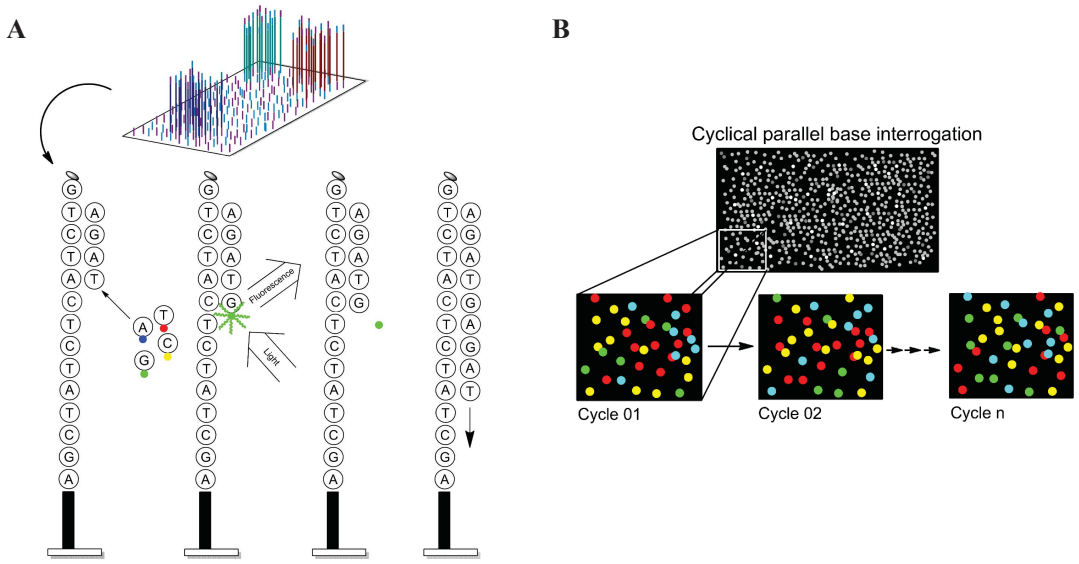


Figure 20 Sequencing-by-synthesis steps (adapted from Morey *et al.*)[270].

A) Sequencing reactions take place in the clonal clusters on the flowcell surface. First, fluorescently-labeled nucleotides are added to the reaction and one nucleotide is incorporated into the growing strand. The fluorescence of the reversible terminator is emitted and registered by the imaging system. Then, the fluorescent dye is cleaved away and the next nucleotide can be incorporated. B) The four colors are detected by total internal reflection fluorescence and can be converted into the corresponding nucleotides.

Bioinformatics workflow

The generation of the huge amount of sequencing data requires a variety of statistical methods and bioinformatics analysis tools[277]. The raw data of a sequencing run is pre-processed into nucleotide base calls in the form of a FASTQ-file. Standard processing includes adapter removal and trimming of the low quality bases at the end of reads[278]. The alignment is the step of matching the short nucleotide reads to a reference genome. Many different tools have been developed for short reads mapping. Alignment tools such as Bowtie2 or BWA are using Burrow-Wheeler Transformation (BWT) compression techniques, Smith-Waterman (SW) Dynamic programing algorithm or the combination of both in order to find the optimal alignment match within an acceptable computational time[279, 280]. Afterwards, variant calling is the process where differences between the reads and the reference genome are identified. Programs such as GATK or SAMtools implement a simple Bayesian model to estimate the likelihood of genotype in the sample based on the observed sequence reads that cover the specific locus[281, 282]. For data visualization, a genome browser is required where the sequencing reads are graphically aligned to a reference genome[270]. Such browsers usually allow the acquisition of annotated data from databases and thus comparative analysis, prediction of pathogenicity of variants, splice-site prediction, expression changes, or protein folding.

1.6.3. Functional studies

According to the guidelines of the American College of Medical Genetics and Genomics (ACMG), the interpretation of sequence variants require well-established *in vitro* or *in vivo* functional studies that support a damaging effect on a gene or protein function[283]. Depending on the function of the protein, different assays can be performed such as immunofluorescence staining for subcellular localization, proliferation assay, plating efficiency assay, Boyden chamber invasion assay, cell-cell adhesion assay, electrophysiological assays or experiments in mice models.

Electrophysiological analysis

The patch-clamp technique permits high-resolution recording of the ionic currents flowing through a cell's plasma membrane[284]. In different configurations, this technique allows to record and manipulate the currents that flow either through single ion channels or those that flow across the whole plasma membrane. In the whole-cell patch-clamp configuration, rupture of the cell membrane is created with a pulse of suction or voltage establishing a low-resistance electrical and physical continuity between the cell and the pipette lumen (Figure 21)[285]. Access to the cell interior allows controlling the voltage of the whole cell via a voltage clamp, observing the current from all the ion channels in the cell membrane, and separating individual current types by controlling the chemical composition on both sides of the cell membrane.

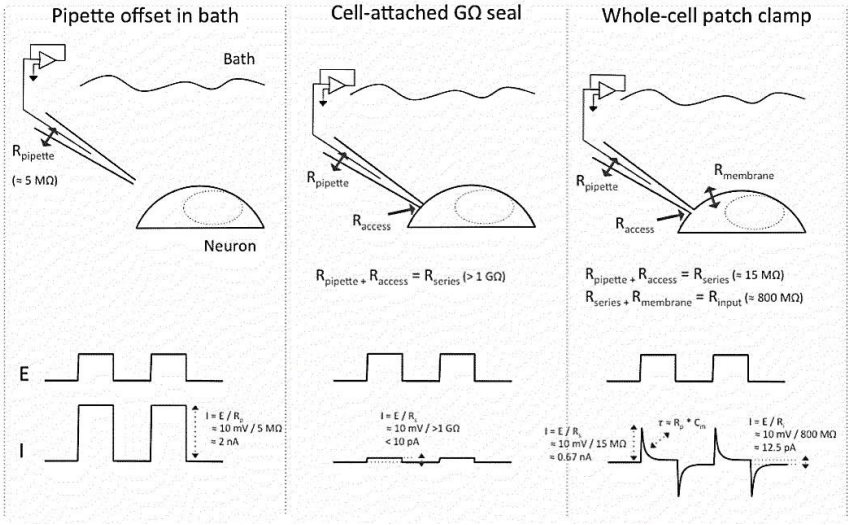


Figure 21 The three main steps of whole-cell patch clamp procedure (copied from www.axolbio.com)

Patch clamp recording uses a fire-polished glass micropipette as a recording electrode and a ground electrode located in the bath. To obtain a high resistance seal, the micropipette is pressed against the surface of a cell and a suction is applied. A portion of the cell membrane is suctioned into the pipette, creating a resistance in the gigaohms range. Another strong pulse of suction leads to the rupture of the membrane and the pipette gains access to the cytoplasm. R = resistance, E = voltage, I = current.

1.7. Aims of the thesis

At the Zurich Institute of Forensic Medicine (ZIFM), around 10-15 sudden death cases per year are reported with no definite explanation about the cause of death despite a complete autopsy procedure, including histology, pharmacological and toxicological screening tests. The vast majority of these cases are SUD victims, whereas sudden death in infants only accounts for around 1% of all cases. Since the early 80ies, alcohol-fixed tissues of SIDS cases have been continuously collected at the ZIFM and were mainly examined by the same forensic pathologist. The cohort of approximately 160 SIDS cases is therefore a very valuable and large cohort compared to international SIDS cohorts.

WES sequencing facilitates the investigation of SIDS/SUD cases in a cost- and time-efficient manner to further understand underlying pathophysiological mechanisms contributing to a sudden death event. The aims of this thesis were: (1) to test whether a WES sequencing approach could be used as an additional tool to investigate the cause of death in SIDS/SUD victims, (2) to develop a variant filtering and classification strategy to identify causative variants within the large amount of variants obtained from sequencing studies and (3) to perform functional studies of potentially disease-causing variants to confirm the pathogenicity of found variants.

II. RESULTS

2.1 Manuscript I

Post-mortem whole exome sequencing (WES) with a focus on cardiac disease-associated genes in five young sudden unexplained death (SUD) cases

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Keywords: sudden unexplained death, cardiac diseases, molecular autopsy, whole-exome sequencing

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Personal contribution: DNA extraction, Sanger sequencing, filtering strategy, variant analysis and variant interpretation, writing the manuscript

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ORIGINAL ARTICLE

Post-mortem whole-exome sequencing (WES) with a focus on cardiac disease-associated genes in five young sudden unexplained death (SUD) cases

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Abstract Sudden death of healthy young adults in the absence of any medical reason is generally categorised as autopsy-negative sudden unexplained death (SUD). Approximately 30 % of all SUD cases can be explained by lethal sequence variants in cardiac genes causing disturbed ion channel functions (channelopathies) or minimal structural heart abnormalities (cardiomyopathies). The aim of this study was to perform whole-exome sequencing (WES) in five young SUD cases in order to identify potentially disease-causing mutations with a focus on 184 genes associated with cardiac diseases or sudden death. WES analysis enabled the identification of damaging-predicted cardiac sequence alterations in three out of five SUD cases. Two SUD victims carried disease-causing variants in long QT syndrome (LQTS)-associated genes (*KCNH2*, *SCN5A*). In a third case, WES identified variants in two genes involved in mitral valve prolapse and thoracic aortic aneurism (*DCHS1*, *TGFβ2*). The genome of a fourth case carried several minor variants involved in arrhythmia pointing to a multigene influence that might have contributed to sudden death. Our results confirm that post-mortem genetic testing in SUD cases in addition to the conventional autopsy can help to identify familial cardiac diseases and can contribute to the identification of genetic risk factors for sudden death.

Keywords Sudden unexplained death · Cardiac diseases · Molecular autopsy · Whole-exome sequencing

Introduction

Sudden death (SD) of a previously healthy adolescent or young adult is a tragic and distressing event for those left behind. Depending on the underlying cause of death, SD can be divided into sudden cardiac death (SCD) or sudden death due to non-cardiac causes such as pulmonary embolism, metabolic, neurological, or infectious conditions [1, 2]. In elderly persons, SCD is mainly caused by coronary artery disease (CAD), whereas in young people (between 1 and 39 years of age), a complete post-mortem examination fails to reveal a cause of death in up to 30 % of all cases [3, 4]. These are generally designated as autopsy-negative sudden unexplained death (SUD) cases. Although SCD is one of the major causes of mortality worldwide, the exact incidence of SUD in young individuals remains unknown in most countries [5, 6].

It is estimated that up to 30 % of all SUD cases carry sequence variants in known cardiac genes causing lethal cardiac arrhythmia in a morphological normal heart [3, 7, 8]. Channelopathies primarily affect the heart rhythm and cardiac electrical conduction including cardiac diseases such as long QT syndrome (LQTS), short QT syndrome (SQTS),

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catecholaminergic polymorphic ventricular tachycardia (CPVT), Brugada syndrome (BrS), and idiopathic ventricular fibrillation (IVF) [9]. Cardiomyopathies on the other hand refer to myocardial disorders characterised by a structural and functional abnormal heart muscle [10]. The most common form is hypertrophic cardiomyopathy (HCM) followed by other cardiomyopathies such as arrhythmogenic right ventricular cardiomyopathy (ARVC), idiopathic dilated cardiomyopathy (DCM), and left ventricular non-compaction cardiomyopathy (LVNC) [11].

Post-mortem genetic testing by using specific gene panels or whole-exome sequencing (WES) represents an efficient tool to elucidate potential disease-causing mechanisms, which remained undetected during autopsy [8, 12, 13]. The exome represents approximately 1–2 % of the human genome, but it accounts for over 85 % of all mutations that have been identified in Mendelian disorders [14]. Loporcaro et al. recently published a first proof-of-principle case report of a WES-based comprehensive molecular autopsy of an otherwise healthy 16-year-old SUDY (SUD in the young) victim, where they identified a pathogenic *MYH7* mutation, previously described in familial HCM, sudden death, and impaired MHC-β actin-translocating and actin-activated ATPase activity [12]. Further studies described the detection of eight ultra-rare variants in seven of 14 Caucasian SUDY victims by focusing on 117 sudden death-susceptibility genes and the identification of likely pathogenic variants in 34 genes in 20 % of forensic SUD cases [8, 15]. Post-mortem genetic analysis of SUD victims may not only enable an accurate determination of cause and manner of death, but may also have significant impact for affected living first-degree family members in whom death-predisposing disorders had remained unrecognised so far [16, 17].

At the Zurich Institute of Forensic Medicine (ZIFM), University of Zurich, Switzerland, approximately 10 sudden death cases in young individuals are reported annually to show no or only minor morphological abnormalities during autopsy, leaving those left behind without any explanation about the exact cause of death. WES is not substantially more expensive than a targeted approach using a gene panel, but allows additional examination of new candidate genes in case of a negative result within the predefined gene list. In this study, we performed WES analyses in five unrelated SUD cases by focusing on 184 cardiac disease-associated genes. We wanted to test whether such a procedure could be implemented as a standard operating procedure to investigate or uncover lethal cardiac diseases.

Material and methods

Five unrelated sudden unexplained death cases (four females and one male) were collected between March and May 2012 at the ZIFM, Switzerland. All SUD cases were examined according to standard forensic procedures, including death scene investigation, a complete autopsy examination with pharmacological-toxicological and histopathological screening, and review of the clinical history. Inclusion criteria were (1) younger than 40 years of age, (2) only minor abnormalities in the heart, and (3) suspicion of an inherited cardiac disease or underlying cardiac arrhythmia contributing to sudden death. DNA of first-degree relatives of the five SUD cases was not available for co-segregation analysis.

Ethical approval

Ethical approval for this study was provided by the local committee in Zurich (KEK-ZH-Nr. 2013–0086), and the study was conducted in full conformance with Swiss laws and regulations.

DNA sample collection and extraction

Five-milliliter EDTA blood samples from the five SUD cases were collected during autopsy and were stored at −20 °C. Genomic DNA was extracted using the Gentra Puregene Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol and quantified using the NanoDrop2000 (Fischer Scientific, Wohlen, Switzerland).

Exome sequencing

Exome capture, sequencing as well as sequence alignment and variant calling were performed at Atlas Biolabs (Berlin, Germany). Nimblegen SeqCap EZ Human Exome Library v.2.0 (Roche NimbleGen Inc., Madison, USA) was used for library preparation, and sequencing was done on the Illumina HiSeq2000 platform (Illumina Inc., San Diego, USA) generating 2 × 100 bp paired-end reads [18]. Alignment of sequence reads, indexing of the reference genome (hg19), and variant calling and annotation was done with a pipeline based on BWA [19], Samtools [20], and Picard and Annovar [21].

Filtering steps and data analysis

Variant filtering included a gene panel of 184 genes associated with cardiac diseases or cardiac death (Supp. Table S1). Annotation of these genes was conducted with the software Alamut HT version 1.1.7 (Interactive Biosoftware, Rouen, France). Output results were imported into an excel-sheet for further analysis. At first, variants were checked manually if they are disease-associated according to entries from the Human Gene Mutation

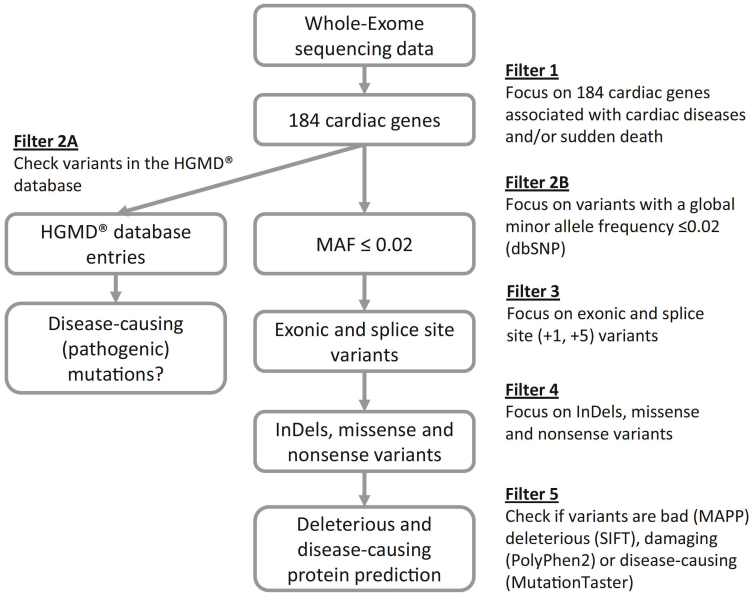

Fig. 1 Filtering flow chart. Whole-exome sequencing data were filtered for 184 genes associated with cardiac diseases and/or sudden death. At first, variants were checked in HGMD® (Human Genome Mutation Database Professional) for reported variant classes (*filter 2A*). The filtering steps for all other variants focused on a global MAF less than 0.02 (*filter 2B*); exonic and splice site variants (*filter 3*); InDels, missense, and nonsense variants (*filter 4*); and deleterious or disease-causing protein prediction (*filter 5*)

Database Professional (HGMD®) (BIOBASE GmbH, Wolfenbüttel, Germany) (Fig. 1). To include known modulator and risk factor variants into data analysis, HGMD®-reported variants were not filtered for a threshold minor allele frequency (MAF). The filtering steps for all other variants focused on (1) a global MAF value less than or equal to 0.02 (based on dbSNP); (2) exonic and splice site (+1, +5) variants; (3) exonic InDels, missense, and nonsense variants; and (4) deleterious or damaging protein predicted functions according to the following in silico predictive algorithms: MAPP (multivariate analysis of protein polymorphism prediction), SIFT (sorting intolerant from tolerant prediction), PolyPhen2, AGVGD (align Grantham-variation Grantham-deviation), and MutationTaster. Sequence alterations at the 5′ donor splice sites +1 and +5 were included into data analysis, because variants at these positions are thought to significantly reduce the pairing of the donor splice site with the complementary site in the small nuclear ribonucleoprotein particle U1snRNP, which is one of the first steps in the complex process of messenger RNA (mRNA) splicing [22]. Alamut Visual version 2.4.7 (Interactive Biosoftware, Rouen, France) was used to visualise the coverage of variants and to review conservation of the variants across a variety of species (based on Ensembl Genome Browser, http://ensembl.org). European allelic frequencies were examined in dbSNP at NCBI (www.ncbi.nlm.nih.gov/projects/SNP/), Ensembl, and in the exome aggregation consortium database (ExAC) (http://exac.broadinstitute.org/).

Possible disease-causing variants must be absent or rare in public databases (dbSNP, ExAC) or in 288 analysed controls (177 males, 111 females) from the general Swiss population.

As a final step, genes identified with disease-causing variants were analysed for splice site and/or synonymous variants.

Confirmation and validation of variants

Potential disease-causing variants found in whole-exome sequencing data of the five SUD cases were confirmed and screened in 288 controls by using standard polymerase chain reaction (PCR) and Sanger sequencing methods (primer sequences available on request). Sequence data were aligned to the reference sequence with the SeqScape® software version v2.6 (Life Technologies, Rotkreuz, Switzerland) (Supplementary data).

Results

Whole-exome sequencing was performed in five unrelated SUD cases. Data were analysed with respect to sequence variants with a focus on 184 genes previously associated with cardiac diseases or cardiac death. WES yielded an average of 87 433 402.8 total reads, and 87.44 % of the target positions were covered at least 30 times (Table 1). Within the exome sequencing data, the five cases had on average 40 658 ± 613.2 single nucleotide variants (SNV) and insertion/deletions (InDels) causing amino acid changes (missense variants) on average in 10 788.4 ± 162.2 variants. By focusing only on the cardiac gene panel, on average 633.2 ± 10.6 variants were obtained per case. After all filtering steps, 23.4 ± 6.3 variants were used for further manual evaluation.

Table 1 Summary of WES data

	Case I	Case II	Case III	Case IV	Case V
Raw data					
Total reads	81′368′374	99′487′232	97′357′972	84′440′830	74′512′606
Variants covered > 10× (%)	97.3	97.6	97.6	97.4	97
Variants covered > 30× (%)	86.6	89.6	89.4	87.5	84.1
Total number of SNV/InDels	40′673	40′647	40′039	41′648	40′283
Exonic variants	23′041	22′908	22′554	23′236	22′621
Missense variants	10′431	10′254	10′155	10′975	10′239
Not reported in dbSNP	1743	958	859	1145	906
Frameshift InDels	170	160	154	155	140
Stopgain	112	105	92	93	83
Stoploss	22	22	19	17	15
Splicing	119	117	119	120	112
Variants in 184 cardiac genes					
Total variants	627	645	620	643	631
HGMD® variants	16	16	21	24	19
MAF ≤ 0.02	130	145	150	137	133
Exonic/splice site variants	75	77	84	87	68
Missense variants	22	23	23	21	17
Not reported in dbSNP	6	4	2	3	1
Deletions	2	0	0	1	0
Insertion	0	0	0	1	0
Nonsense	0	0	2	0	0

MAF minor allele frequency, *SNV* single nucleotide variants, *InDels* insertion/deletions

Case I

Case I was a 27-year-old woman (weight, 54.1 kg; height, 172 cm; European origin) found dead on a sofa by her roommate late in the evening. Autopsy investigations showed a normal heart size (330 g) according to Zeek [23], but a swollen and oedematous mitral valve with connective tissue changes in papillary muscles and thickening of tendinous chords (Supp. Fig. S1a). Histological examination of the mitral valve revealed thickening and mucoid swelling of the tissue with signs of collagenous destruction (Supp. Fig. S1b). Microbiological, virological, and pharmacological-toxicological screening results were all negative and showed no signs of an acute inflammation prior to death.

At least two potential disease-causing heterozygous variants were detected in the 27-year-old woman (Table 2). A missense variant was located in the dachsous cadherin-related 1 gene (*DCHS1*; NM_003737.2: c.3415C > T: p.Arg1139Cys) with disease-causing protein function predictions and an allele frequency of 3.7e-05 in the general population (ExAC). The second variant was a one nucleotide, frame-shifting deletion in the transforming growth factor beta 2 gene (*TGFβ2*; NM_001135599.2: c.440delC: p.Pro.147 Argfs*27) leading to a truncated protein, not previously described in respective databases or in the scientific literature. The ancestral alleles of both variants were highly conserved during evolution.

Case II

Case II was a 30-year-old woman (weight, 51.6 kg; height, 166 cm; European origin). She collapsed in the bathroom and was brought into her bed, where she died shortly afterwards. She had severe depression and was alcohol-dependent. Chemical screening tests detected a massively increased amount of butyric acid (almost a lethal concentration of 2500 μmol/L) and further pathological metabolic products such as acetone. Pharmacological-toxicological screening tests detected therapeutic concentrations of benzodiazepines in the peripheral blood. Autopsy investigations showed a heart weight of 390 g (24 % enlarged according to Zeek) with an adipose right ventricle and focally enlarged myocytes.

Within the 184 cardiac genes, we found at least two important possibly phenotype-relevant heterozygous missense variants (Table 2). One variant (rs76906304) was located in the gap junction protein delta 4 gene (*GJD4*; NM_153368.2: c.259C > T: p.Leu87Phe). The second variant was detected in the junctophilin type 2 gene (*JPH2*; NM_020433.4: c.1013A > G: p.Glu338Gly), not previously described in the

Table 2 Potential disease-causing variants in cases I–V

Case	Gene	RefSeq	dbSNP	Exon	Coding effect	Nucleotide change	Protein change	HGMD®-Phenotype	HGMD® variant class	MAPP
I	*DCHS1*	NM_003737.3	–	6	Missense	c.3415C > T	p.Arg1139Cys	–	–	Bad
	TGFβ2	NM_001135599.2	–	3	Frameshift	c.440delC	p.Pro147Argfs*27	–	–	NA
II	*GJD4*	NM_153368.2	rs76906304	2	Missense	c.259C > T	p.Leu87Phe	–	–	Bad
	JPH2	NM_020433.4	–	2	Missense	c.1013A > G	p.Glu338Gly	–	–	Bad
III	*DSP*	NM_004415.2	rs78652302	24	Missense	c.5498A > T	p.Glu1833Val	Cardiomyopathy	DM?	Bad
	KCNH2	NM_000238.3	–	13	Nonsense	c.3002G > A	p.Trp1001*	LQTS	DM	NA
	MYH7	NM_000257.3	rs3729823	32	Missense	c.4472C > G	p.Ser1491Cys	Cardiomyopathy	DM?	Bad
	RANGRF	NM_016492.4	rs140704891	2	Nonsense	c.181G > T	p.Glu61*	Altered function	FP	NA
IV	*DSP*	NM_004415.2	rs28763971	24	Missense	c.8605A > G	p.Ile2869Val	–	–	Bad
	KCNQ1	NM_000218.2	rs12720449	11	Missense	c.1343C > G	p.Pro448Arg	LQTS	DM?	Unknown
	KCNH2	NM_000238.3	rs1805123	11	Missense	c.2690A > C	p.Lys897Thr	Atrial fibrillation	DFP	Bad
	SCN10A	NM_001293306.2	rs148041371	18	Missense	c.3291C > G	p.Ile1097Met	–	–	Bad
V	*SCN5A*	NM_001099404.1	–	28	Missense	c.5320A > C	p.Asn1774His	–	–	Bad
	SCN5A	NM_001099404.1	rs1805124	12	Missense	c.1673A > G	p.His558Arg	Phenotype modifier	DFP	Tolerated

Case	SIFT	Polyphen2	AGVGD class	Mutation taster	MAF (dbSNP)	MAF (ExAC)	MAF (in-house controls)	Nr. of reads in WES
I	Deleterious	Probably damaging	C45	Disease-causing	NA	0.000	0.000	121
	NA	NA	NA	Disease-causing	NA	NA	0.000	61
II	Deleterious	Probably damaging	C15	Disease-causing	0.005	0.013	NA	94
	Deleterious	Damaging	C65	Disease-causing	NA	NA	NA	35
III	Deleterious	Probably damaging	C65	Disease-causing	0.008	0.009	NA	65
	NA	NA	NA	Disease-causing	NA	NA	0.000	6
	Deleterious	Benign	C0	Disease-causing	0.008	0.007	NA	75
	NA	NA	NA	Disease-causing	0.002	0.004	NA	39
IV	Deleterious	Benign	C0	Disease-causing	0.006	0.002	NA	10
	Tolerated	Benign	C0	Polymorphisms	0.000	0.007	NA	41
	Deleterious	Benign	C0	Disease-causing	0.129	0.187	NA	21
	Deleterious	Probably damaging	C0	Disease-causing	0.005	0.001	NA	63
V	Deleterious	Probably damaging	C65	Disease-causing	NA	NA	0.000	120
	Tolerated	Benign	C0	Polymorphism	0.206	0.221	NA	71

DM? disease-causing mutation?, *DM* disease-causing mutation, *DP* disease-associated polymorphisms reported to be in significant association with diseases and assumed to be functional, *FP* in vitro or in vivo functional polymorphisms, *FP* in vitro or in vivo functional polymorphisms, *DFP* disease-associated polymorphisms with additional supporting functional evidence, *MAF* minor allele frequency, *NA* not available

literature or public databases. Both variants were predicted to be disease-causing/damaging according to the in silico predictive algorithms.

Case III

Case III was a 31-year-old man (weight, 87.6 kg; height, 186 cm; European origin) who was found dead sitting on the toilet in his apartment. He worked in a restaurant kitchen, but did not appear to his working shift although he was seen shortly before and was considered to be reliable. On the evening before, he has attended a concert with some colleagues where they allegedly consumed different drugs. The autopsy showed a moderate enlarged heart (390 g) without further morphological abnormalities. Post-mortem pharmacological-toxicological screening results were positive for cannabis and amphetamine. Further investigations of the medical history revealed a long QT syndrome (LQTS) diagnosis which was already detected in his adolescence based on electrocardiography (ECG) results (Supp. Fig. S2). His physician recommended an annual medical check including an ECG stress test, but he did not prescribe any medications. Two of the deceased's brothers and mother had the same diagnosis of LQTS, but without any implications so far (Supp. Fig. S3).

Within the cardiac gene panel, we found two nonsense alterations and two missense variants (Table 2). One stop variant (rs121912509) was detected in the gene encoding the potassium voltage-gated channel, subfamily H, member 2 (*KCHN2*; NM_000238.3: c.3002G>A: p.Trp1001*), reported as a LQT2 disease-causing mutation according to HGMD®. Due to the low number of reads (coverage=6) at this position, Sanger sequencing was performed to confirm the variant (supplementary data). A second stop variant (rs140704891) was identified in the RAN guanine nucleotide release factor gene (*RANGRF*; NM_016492.4: c.181G>T: p.glu61*) and is classified as an in vitro or in vivo functional polymorphism in HGMD®. Two potentially disease-relevant heterozygous missense variants were located in the desmoplakin gene (*DSP*; NM_004415.2: rs78652302: c.5498A>T: p.Glu1833Val) and in the myosin heavy chain 7 gene (*MYH7*; NM_000257.2: rs3729823: c.4472C>G: p.Ser1491Cys). Both variants were described as possible disease-causing mutations according to HGMD® (rs78652302: dilated cardiomyopathy and rs3729823: hypertrophic cardiomyopathy), and protein effects were predicted to be deleterious (SIFT), probably damaging (polyphen2), and disease-causing (MutationTaster). MAF for both variants is ~1 % in the general population, but shows a variable frequency within the European population (Ensembl: rs78652302: 1–3%T and rs3729823: 0–3 %G).

Case IV

Case IV was a 38-year-old woman (weight, 58.8 kg; height, 162 cm; Tibetan origin). She was found dead in the morning by her 10-year-old daughter who was sleeping in the same bed. Since several weeks, the woman complained about the air conditioner at her new job as she had a cold, but was otherwise healthy. Autopsy findings showed a normal heart size (250 g) and a slightly fatty infiltrated right ventricle without any further morphological abnormalities. Family history was negative for cardiac diseases or sudden death.

Genetic testing identified no rare disease-causing sequence variants within the cardiac gene panel, but the woman had several minor variants which probably affect function (Table 2). One heterozygous missense variant was located in *KCNH2* (rs1805123: c.2690 T>G: p.Lys897Thr) and is associated with atrial fibrillation and drug-induced arrhythmia according to HGMD®. Although the minor allele is common in the European population (20%G), the variant is significantly less common in the Asian population (4%G). Further heterozygous missense variants were located in *DSP* (rs28763971: c.8605A>G: p.Ille2869Val), in the potassium voltage-gated channel, KQT-type gene (*KCNQ1*; NM_000218.2: rs12720449: c.1343C>G: p.Pro448Arg), and in the sodium channel type 10 gene (*SCN10A*; NM_006514.2: rs148041371: c.3291C>G: p.Ille1097Met). Although all three sequence variants are very rare in the European population, they are much more common in the Asian population (Ensembl: rs28763971: 2%G; rs12720449: 11%G; rs148041371: 2%G).

Case V

Case V was a 19-year-old woman (weight, 68.4 kg; height, 170 cm; European origin) who gave birth to her first child 2 months before she died. She had a minor cough, but was otherwise healthy when she suddenly collapsed at her home. The ambulance transferred her to the hospital where life-sustaining measures were stopped after the diagnosis of myocardial infarction and irreversible brain damage. Autopsy investigation showed a broadening and fatty infiltrated right ventricle possibly indicating ARVC (Supp. Fig. S4 a, b), and the heart was slightly enlarged (300 g). Pharmacological-toxicological screening tests were negative for drugs and alcohol. Further investigations of the family history showed that a niece of the deceased died suddenly at the age of 11 during sports, and the grandfather died suddenly while sweeping snow at the of age of 77 (Supp. Fig.S5). Furthermore, her mother had minimal prolongation of the heart rate corrected QT intervals (QTc) of 460–480 ms [24]; however, she did not follow further medical examinations. The toddler of this young woman has been under medical observation, a QTc

prolongation (495 ms) has been detected during sleep, but the echocardiogram was normal.

Analysis of sequence variants within the 184 cardiac genes revealed two heterozygous variants in the voltage-gated sodium channel type V, alpha subunit (*SCN5A*) (Table 2). One missense variant was located in exon 28 (NM_001099404.1: c.5320A > C, p.Asn1774His) and was not previously reported in the literature and in public databases. A second missense variant was located in exon 12 (NM_001099404.1: rs1805124, c.1673A > G, p.His558Arg) and is described as disease-associated polymorphism with additional supporting functional evidence according to HGMD®. However, prediction algorithms described the variant as tolerated, and MAF is 21 % in the general population.

Discussion

Molecular testing in autopsy-negative death cases can identify the underlying mechanisms for sudden death and may represent an efficient tool in combination with standard forensic investigations to identify or confirm the suspicion of an inherited sudden death-predisposing disorder [8].

In this study, genetic testing by using WES enabled the identification of potential lethal cardiac disease-associated alterations in three out of five SUD victims (Table 3). A fourth case carried several minor variants pointing to a multigene influence instead of a strong autosomal-dominant sequence variant which could explain the phenotype.

Case I had one missense variant in *DCHS1* (p.Arg1139Cys) and a one nucleotide frame-shift deletion in *TGFβ2* (p.Pro.147 fs*27). Sequence variants in both genes were already reported in mitral valve prolapse (MVP), which is a valvular heart disease characterised by the displacement of an abnormally thickened mitral valve leaflet into the left atrium during systole leading to serious complications, including arrhythmia, heart failure, and sudden death [25]. Reanalysis of the case report showed that the young woman had scoliosis and was very tall with long, slender limbs and fingers. Point mutations and whole-gene deletions/duplications in *TGFβ2* are reported in patients with Loeys-Dietz syndrome (LDS), familial thoracic aortic aneurysms, and acute aortic dissection (TAAD) associated with mild systemic features of Marfan syndrome (MFS) [26]. Phenotypical features and genetic testing strongly indicated that the young woman had an underlying connective tissue disease, obviously not diagnosed during her lifetime, which might have contributed to mitral valve prolapse leading to the sudden death of this young woman.

WES analysis in case II identified two variants in *GJD4* (p.Leu87Phe) and *JPH2* (p.Glu338Gly). Connexins, such as GJD4, are transmembrane proteins involved in the formation of gap junction channels in the heart. Alterations in the connexin family are associated with cardiac arrhythmias and sudden infant death syndrome (SIDS) [27, 28]. Junctophilin type 2 is a cardiac member of the junctional membrane complex proteins, and JPH2 knockout mice demonstrated embryonic lethality and irregular calcium handling suggesting a crucial role for junctophilin-2 in facilitating intracellular calcium release and cardiac contractility [29]. Sequence variants in *JPH2* were reported in atrial fibrillation due to impaired ryanodine receptor Ca^{2+} channels (RyR2) stabilisation and in familial HCM [30]. Although the primary cause of death in this young woman was already diagnosed due to metabolic imbalance, the two *GJD4* and *JPH2* variants could have contributed to the morphological abnormalities in her heart, triggering the sudden death event during a critical time period.

Case III carried two nonsense sequence variants in *RANGRF* (p.glu61*) and *KCNH2* (p.Trp1001*). RANGRF protein regulates the expression and function of SCN5A (Nav1.5) cardiac sodium channel. Electrophysiological studies in CHO-K1 cells demonstrated that the p.Glu61* stop completely failed to increase the sodium channel current compared to wild-type and may increase the risk of arrhythmias [31]. The Nav1.5 interacting part of RANGRF is positioned in the amino acid range 65–186 and hence a truncated protein produced from the affected allele would presumably not bind to the Nav1.5 leading to a loss of function [32]. *KCNH2* encodes the pore forming subunit of the rapidly activating delayed rectifier K^+ channel (I_{Kr}) in the heart. Sequence variants are associated with delayed cardiac repolarization and prolonged QTc intervals leading to ventricular arrhythmias and sudden death [33]. The stop variant (c.3002C > T; p.Trp1001*) affects the C-terminus region of the KCNH2 protein leading to assembly abnormality caused by a reduction of mutant mRNA transcripts by nonsense-mediated mRNA decay [34]. The p.Trp1001* could be the major underlying pathogenic sequence alteration causing QTc interval prolongation in this young man, confirming the ECG-based clinical diagnosis of LQTS. In addition, QTc interval prolongation is described in patients taking amphetamine-type stimulants [35], also detected in therapeutic concentration in this 31-year-old man.

Genetic testing in case IV identified one variant in *KCNH2* (p.Lys897Thr) and several borderline variants in arrhythmia-associated genes (*DSP*: p.Ille2869Val; *KCNQ1*: p.Pro448Arg; *SCN10A*: p.Ille1097Met). Although sequence variants in *KCNH2* are described in drug-induced QTc prolongation [36], we have no evidence if the woman had taken any medications against her cold prior to death. The common p.Lys897Thr is associated with an increased risk of developing torsade de pointes in the subacute phase of myocardial infarction and is described as a genetic modifier of latent congenital LQTS [37, 38]. Furthermore, cases with multiple borderline variants are more susceptible to develop diseases, even in the absence of strong dominant mutations that explain the phenotype. One major problem during data analysis was that

Table 3 Summary of autopsy and genetic findings in the five SUD cases

Case	Gender	Age (years)	Autopsy findings	Toxicological screening	Personal history	Family history	Genetic findings	Presumed cause of death based on autopsy and genetic findings
I	Female	27	Normal heart size (330 g) Swollen and oedematous mitral valve Scoliosis Very tall Long limbs/fingers	Negative	NA	NA	*DCHS1* p.Arg1139Cys *TGFβ2* p.Pro147Argfs*27	Marfan syndrome Mitral valve prolapse
II	Female	30	Enlarged heart (390 g) Focally enlarged myocytes	Benzodiazepines Alcohol	Severe depression Alcohol-dependent	NA	*GJD4* p.Leu87Phe *JPH2* p.Glu338Gly	Metabolic imbalance
III	Male	31	Moderate enlarged heart (390 g)	Cannabis Amphetamine	Medical history of LQTS type 2 Prolonged QTc interval (520 ms)	Two brothers and mother diagnosed with LQTS	*DSP* p.Glu1833Val *KCNH2* p.Trp1001* *MYH7* p.Ser1491Cys *RANGRF* p.Glu61*	LQTS
IV	Female	38	Normal heart size (250 g) Slightly fatty infiltrated right ventricle	Negative	Cold since several weeks	No SD in family	*DSP* p.Ile2869Val *KCNQ1* p.Pro448Arg *KCNH2* p.Lys897Thr *SCN10A* p.Ile1097Met	Remained unexplained
V	Female	19	Slightly enlarged heart size (300 g) Fat-infiltrated right ventricle ARVC suspicion	Negative	Minor cough Gave birth 2 months before her death	Maternal history of SD Mother and son diagnosed with prolonged QTc intervals	*SCN5A* p.Asn1774His *SCN5A* p.His558Arg	ARVC LQTS

ARVC arrhythmogenic right ventricle cardiomyopathy, *NA* not available, *SD* sudden death

the allelic frequencies of Tibetan origin were not available in public databases, complicating accurate interpretation of the found sequence variants.

Case V carried a missense variant in *SCN5A* (p.Asn1774His) not previously reported in public databases. Kato et al. described a 1-day-old boy with prolonged QTc intervals (680 ms) having asparagine to aspartic acid replacement at the same position (rs199473633, c.5320 T > C, p.Asn1774Asp), and his brother died suddenly in infancy [39]. A functional assay demonstrated that p.Asn1774Asp channels carried significantly larger late components of sodium current than wild-type cells. The same amino acid replacement (p.Asn1774Asp) was also reported in a male foetus, which showed typical clinical signs of LQT3 (convulsion, ventricular tachycardia, torsade de pointes, atrioventricular block, and QTc intervals of 670 ms) [40]. To investigate the consequences of the asparagine to histidine amino acid change found in this young woman, functional studies are required in order to study the biological relevance of this substitution. The second missense variant in *SCN5A* (p.His558Arg) is described as phenotype modifier in LQTS and might amplify or modulate the functional electrophysiological impairment of genetic variants located in the same gene [41, 42]. Genetic analysis could not confirm a suspected ARVC although histopathological findings indicated clear fibro-fatty tissue infiltration with replacement of the myocardium in the right ventricle. Approximately 50 % of ARVC cases remain negative after genetic testing. This might be explained by the complex genetic background of ARVC and low penetrance making it difficult to identify the true disease-causing variants accurately [43]. In summary, morphological abnormalities in the heart led to an ARVC suspicion during autopsy; however, genetic testing could not confirm this suspicion but indicated a possible disturbed ion channel function. In the end, both of them could have contributed to cardiac arrhythmias triggering her lethal cardiac infarction.

WES is one of the most commonly used new sequencing approaches and major advantages are a high throughput, well established sample preparation methods, and relatively low preparation and sequencing costs compared to traditional Sanger sequencing [44]. Additionally, sample preparation and sequencing can be performed from a small input amount of DNA, which can be crucial for post-mortem samples where the amount of available tissue and the DNA quality are limited. Library preparation and sequencing costs are comparable to a targeted gene approach, but the WES data analysis can be extended to the whole exome in order to identify new candidate genes in cases where the analysis remained negative within a predefined gene panel.

Significant challenges in WES represent the handling and processing steps of such huge amount of sequencing data, which requires a variety of computational and statistical approaches [45]. Consequently, sequencing data analysis and mainly interpretation are time-consuming and require expertise in the field to prevent false-positive or false-negative results. Although filtering strategies and protein prediction tools can help to interpret the pathogenicity of variants, pathological and functional consequences of variants often remain unclear and require further functional studies [46]. Negative genetic testing otherwise may be secondary to disease-causing sequence variants within genes not included in the cardiac gene panel, due to sequence alterations not detectable in WES approaches, such as deep intronic or splice mutations, or due to variants lost during bioinformatics steps rather than due to real absence of any sequence alterations. Accurate interpretation is even more difficult in complex multifactorial phenotypes such as in sudden unexplained death cases without any obvious morphological abnormalities, and lethality may be triggered by different events such as stress, drugs, or physical exertion [47].

The legal and ethical aspects of genetic analysis in forensic post-mortem investigations are complex, as the main forensic purpose is to determine the cause of death and not its implications for surviving relatives [48]. Therefore, genetic counselling of family members should be based on a multidisciplinary approach involving forensic pathologists, geneticists, and cardiologists to provide the family an opportunity to be informed in case of positive findings and offering them further clinical evaluation. Nowadays, minor preventive measures such as lifestyle modifications, prophylaxis, drug therapy, and implantable defibrillators are available in most cardiac diseases preventing another tragedy within the family [2]. In this study, genetic counselling of first-degree relatives was performed in two of the cases with potential disease-causing variants (case III and case V), whereas the relatives of the other three cases were not available for counselling. Furthermore, functional studies to confirm the pathogenicity of found variants and family co-segregation analysis would have been crucial to verify the possible disease-causing impact of the observed variants, which are potential limitations of this study. DNA from family members of the five SUD cases was not available for co-segregation analysis, and hence, it is unknown if the observed variants were inherited from the parents or occurred de novo in the deceased.

In general, post-mortem genetic testing demonstrates an efficient strategy to investigate the cause of death in autopsy-negative sudden death cases. However, one of the major challenges remains the data management and variant interpretation of the huge amount of exome sequencing data and results should be regarded with caution.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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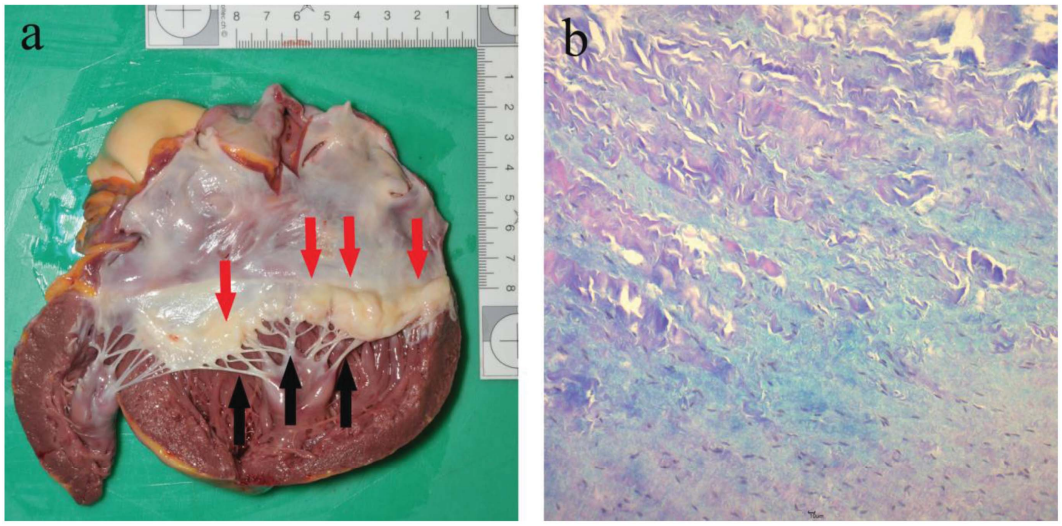
Supplementary Information

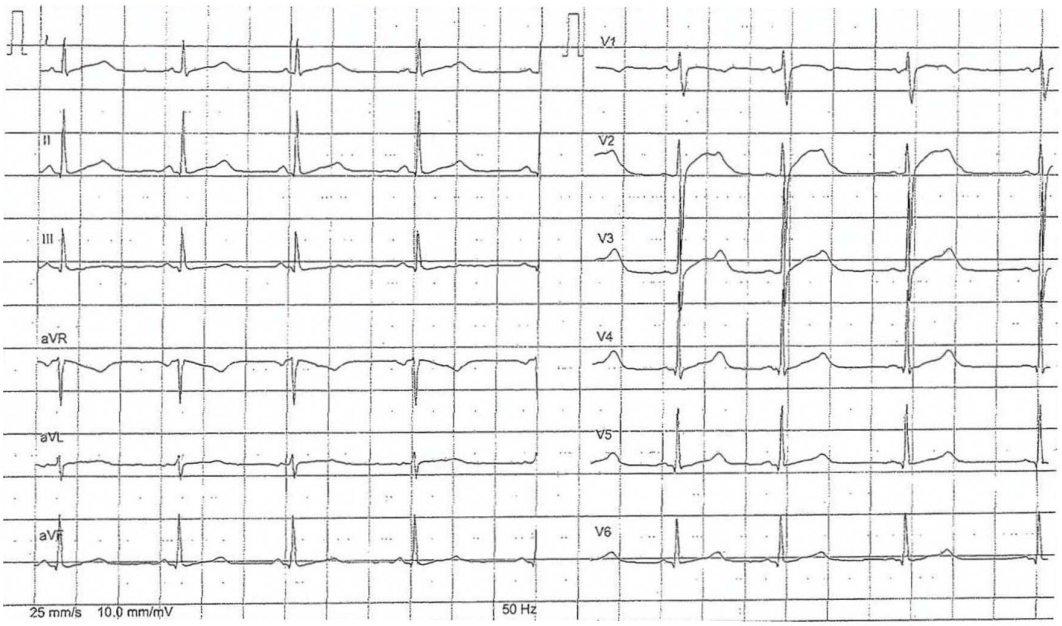
Neubauer et al., Post-mortem whole-exome sequencing (WES) with a focus on cardiac disease-associated genes in five young sudden unexplained death (SUD) cases

Int J Legal Med doi.10.1007/s00414-016-1317-4



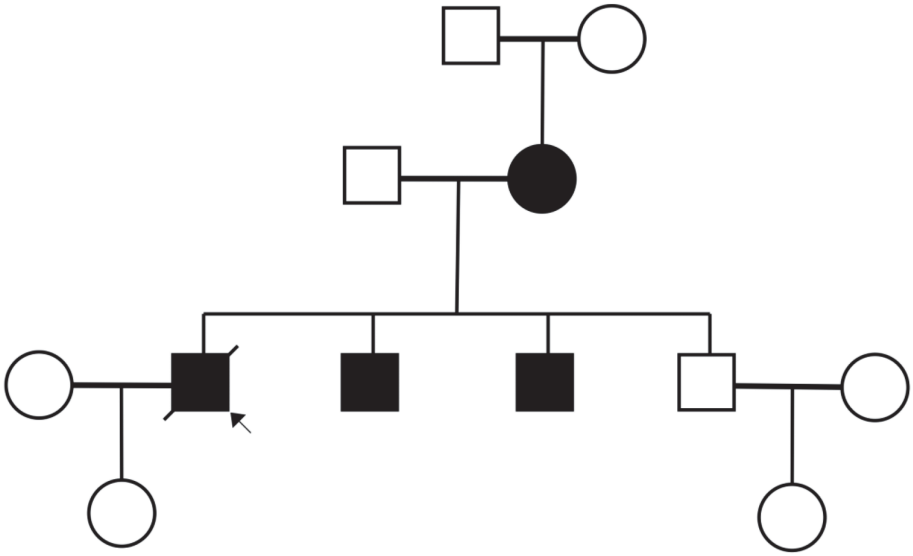
Supp. Fig. S1 Post-mortal data of case I

a Sliced mitral valve with oedematous swollen leaflets (red arrows) and thickening of chordae tendineae (black arrows). **b** Alcian blue-PAS staining of the mitral valve (tenfold enlargement) showing mucoid swelling of the tissue with signs of collagenous destruction.



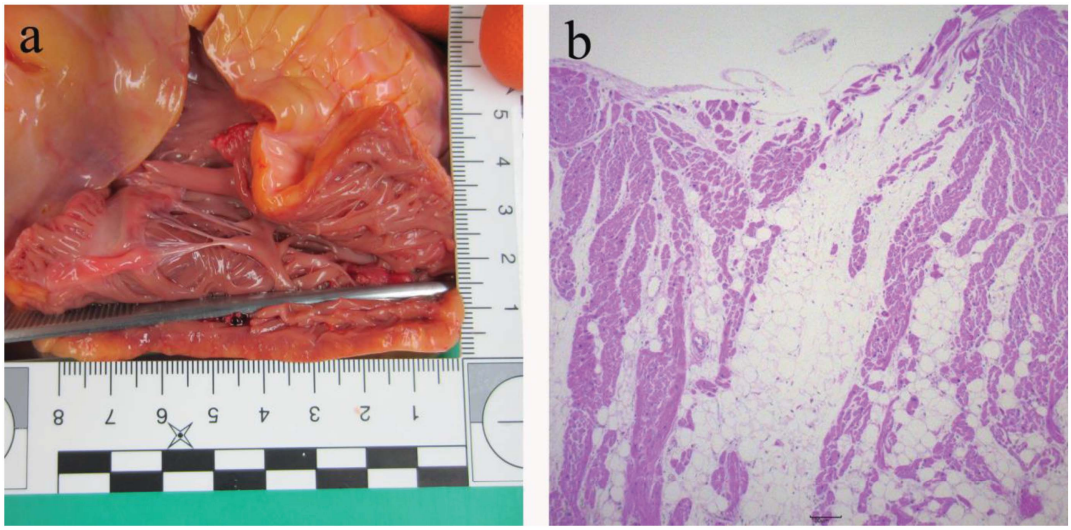
Supp. Fig. S2 Electrocardiogram of case III

12-lead electrocardiogram obtained previously on this patient showing bradycardia sinus rhythm, heart rate 53x min, notched T waves (typical in LQTS2) and abnormal QTc of 520 msec.



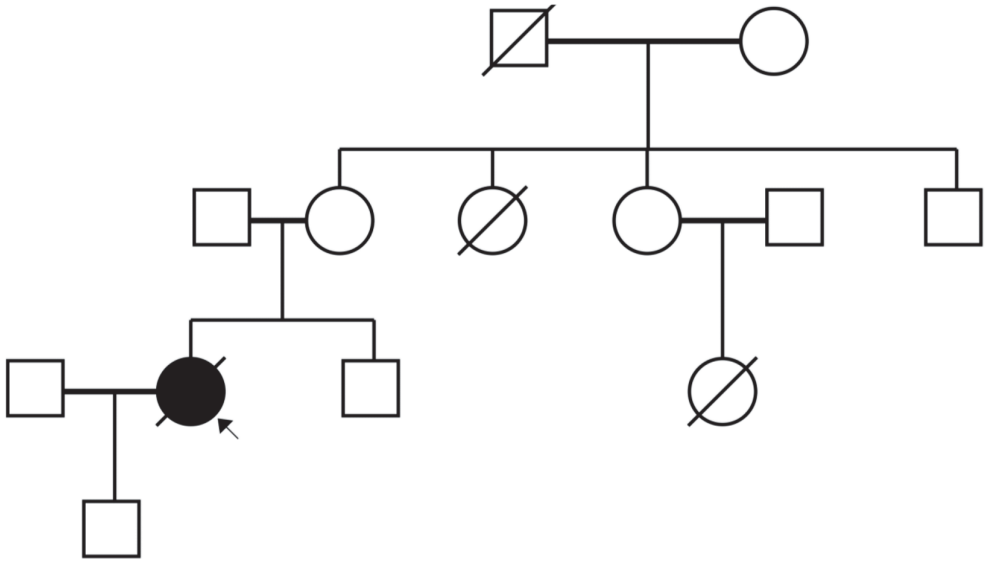
Supp. Fig. S3 Pedigree of case III

The 31-years-old index case is indicated with an arrow. Two of his brother's and his mother had the same QTc-prolonged diagnosis based on ECG, but are without any implications so far. The grandfather of the deceased had a myocardial infarction with 50 years of age.



Supp. Fig. S4 Post-mortal data of case V

a Sliced right ventricle showing fatty infiltration and rarefied myocardium. **b** Haematoxylin-eosin staining of the right ventricle showing massively fatty infiltration with loss of myocardium.



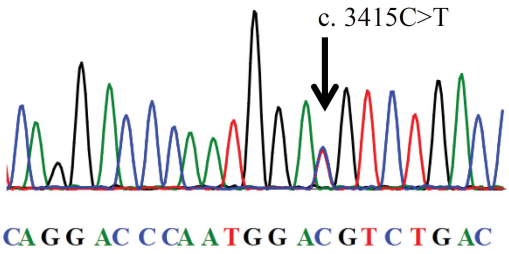
Supp. Fig. S5 Pedigree of case V

The 10-year-olf index case is indicated with an arrow. The niece of the 19-years-old deceased died suddenly at the age of 11 during sports and her grandfather died suddenly while sweeping snow at the age of 77. Furthermore, the mother and the toddler of the young woman were diagnosed with minimal QTc prolongations.

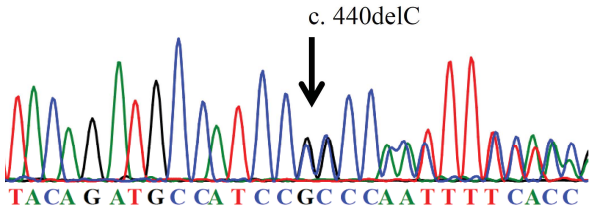
Sanger Sequencing variant confirmation for the most relevant disease-causing variants in SUD cases

Case I

DCHS1 NM_003737.2, c.3415C>T, p.Arg1139Cys

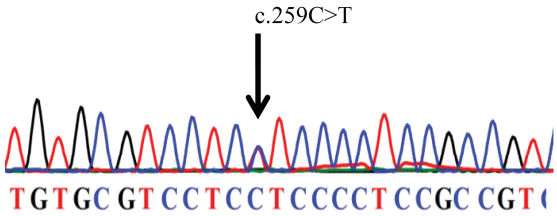


TGFβ2 NM_00113599.2 c.440delC, p.Pro147Argfs*27



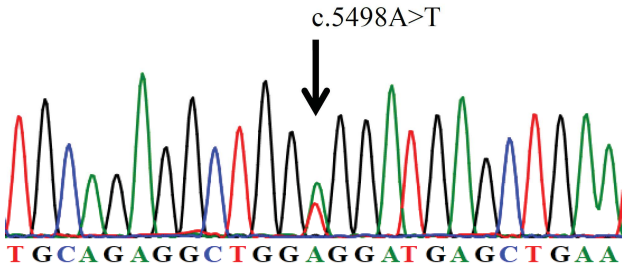
Case II

GJD4 NM_153368.2 rs76906304, c.259C>T, p.Leu87Phe

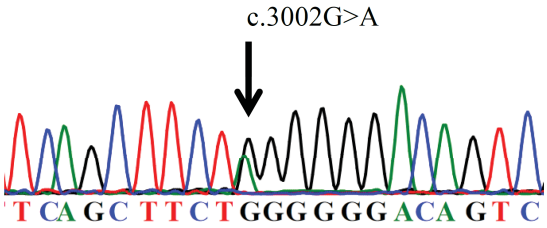


Case III

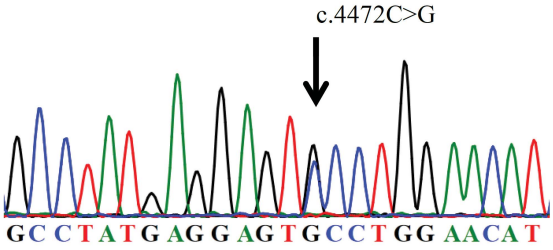
DSP NM_004415.2, rs78652302, c.5498A>T, p.Glu1833Val



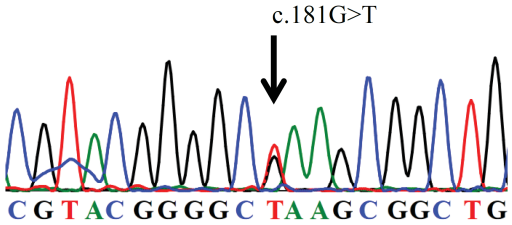
KCNH2 NM_000238.3, c.3002G>A, p.Trp1000*



MYH7 NM_000257.2, rs3729823, c.4472C>T, p.Ser1491Cys

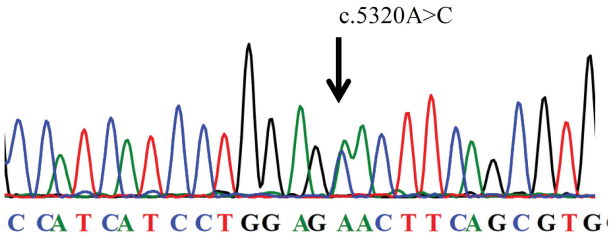


RANGRF NM_016492.4, rs140704891, c.181C>T, p.Glu61*



Case V

SCN5A NM_001099404.1, c.5320A>C, p.Asn1774His



Supp Data Table 1 List of genes (part 1).

Gene	Protein name	RefSeq-Nr.	Disease association
ABCC8	ATP-binding cassette, subfamily C (CFTR/MRP), member 8	NM_001287174.1	IVF
ABCC9	ATP-binding cassette, subfamily C (CFTR/MRP), member 9	NM_005691.3	Afib, Cantu Syndrome, DCM.
ACADVL	Acyl-CoA dehydrogenase, very long chain	NM_001270447.1	DCM, HCM
ACTA2	Actin, alpha 2, smooth muscle, aorta	NM_001141945.1	Aneurysm
ACTC1	Actin, alpha, cardiac muscle 1	NM_005159.4	ASD, DCM, HCM
ACTN2	Actinin, alpha 2	NM_001103.3	DCM, HCM
ACVRL1	Activin A receptor type II-like 1	NM_000020.2	PAH
ADAMTS10	ADAM metallopeptidase with thrombospondin type 1 motif 10	NM_030957.3	Weill-Marchesani
AGL	Amylo-alpha-1,6-glucosidase, 4-alpha-glucanontransferase	NM_000028.2	HCM
AKAP9	A kinase (PRKA) anchor protein (yotiao) 9	NM_001148.4	LQTS
ANK2	Ankyrin 2	NM_001148.4	CPVT, LQTS
ANKRD1	Ankyrin repeat domain 1 (cardiac muscle)	NM_014391.2	DCM, HCM
ATP5E	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	NM_006886.3	HCM
BAG3	Bcl2-associated athanogene 3	NM_004281.3	DCM
BMPR1B	Bone morphogenetic protein receptor, type IB	NM_001256793.1	PAH
BMPR2	Bone morphogenetic protein receptor, type II	NM_001204.6	PAH
BRAF	V-raf murine sarcoma viral oncogene homolog B	NM_004333.4	HCM
CACNA1C	Calcium channel, voltage-dependent, L type, a 1C subunit	NM_199460.3	BrS, LQTS, SQTS
CACNA2D1	Calcium channel, voltage-dependent, a 2/d subunit 1	NM_000722.2	BrS, SQTS
CACNB2B	Calcium channel, voltage-dependent, b 2 subunit	NM_000724.3	BrS, SQTS
CALM1	Calmodulin 1	NM_006888.4	CPVT, LQTS
CALM2	Calmodulin 2	NM_001743.4	LQTS
CALM3	Calmodulin 3	NM_005184.2	CPVT, IVF, LQTS
CALR3	Calreticulin 3	NM_145046.4	HCM
CAMK2G	Calcium/calmodulin-dependent protein kinase II gamma	NM_172171.2	Afib
CASQ2	Calsequestrin 2 (cardiac muscle)	NM_001232.3	CPVT, LVNC
CAV1	Caveolin 1	NM_001753.4	PAH
CAV3	Caveolin 3	NM_001234.4	HCM, LQTS
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase	NM_005188.3	Noonan-syndrome
COA5	Cytochrome c oxidase assembly factor 5	NM_001008215.2	HCM
COL3A1	Collagen, type III, alpha 1	NM_000090.3	Ehrles Danlos syndrome
COL5A1	Collagen, type V, alpha 1	NM_000093.4	AA
COL5A2	Collagen, type V, alpha 2	NM_000393.3	AA
CRYAB	Crystallin, a B	NM_001289807.1	DCM, HCM
CSRP3	Cysteine- and glycine-rich protein 3 (cardiac LIM protein)	NM_003476.4	DCM, HCM

Supp Data Table 1 List of genes (part 2).

Gene	Protein name	RefSeq-Nr.	Disease association
CTF1	Cardiotrophin 1	NM_001330.3	DCM
CTGF	Connective tissue growth factor	NM_001901.2	HCM
CTNNA3	Catenin (cadherin-associated protein), alpha 3	NM_001127384.2	ARVC
DCHS1	Dachsous cadherin-related 1	NM_003737.3	MVP
DES	Desmin	NM_001927.3	ARVC, DCM, HCM
DLG1	Discs, large homolog 1	NM_004087.2	LQTS
DMD	Dystrophin, muscular dystrophy	NM_004006.2	DCM
DMPK	Dystrophia myotonica-protein kinase	NM_001288764.1	DCM
DNAJC19	DNAJ (Hsp40) homolog, subfamily C, member 19	NM_145261.3	DCM, LVNC
DNM1L	Dynamin 1-like	NM_001278464.1	DCM
DOLK	Dolichol kinase	NM_014908.3	DCM
DPP6	Dipeptidyl-peptidase 6	NM_130797.3	IVF
DSC2	Desmocollin 2	NM_024422.4	ARVC, DCM
DSG2	Desmoglein 2	NM_001943.3	ARVC, DCM
DSP	Desmoplakin	NM_004415.2	ARVC
DTNA	Dystrobrevin, alpha	NM_001390.4	LVNC
EFEMP2	EGF containing fibulin-like extracellular matrix protein 2	NM_016938.4	AA, Cutis Laxa syndrome
ELN	Elastin	NM_001278939.1	Cutis Laxia syndrome
EMD	Emerin (Emery-Dreifuss muscular dystrophy)	NM_000117.2	DCM
ENG	Endoglin	NM_001114753.2	PAH
EYA4	Eyes absent homolog 4 (Drosophila)	NM_172105.3	DCM
FBN1	Fibrillin 1	NM_000138.4	Marfan syndrome, AA
FBN2	Fibrillin 2	NM_001999.3	Marfan syndrome, AA
FGF12	Fibroblast growth factor 12	NM_021032.4	BrS
FHL1	Four and a half LIM domains 1	NM_001159702.2	HCM
FHL2	Four and a half LIM domains 2	NM_001450.3	DCM
FKTN	Fukuyama-type congenital muscular dystrophy (fukutin)1	NM_006731.2	DCM
FLNA	Filamin A, alpha	NM_001110556.1	MVP, TAAD
FOXRED1	FAD-dependent oxidoreductase domain containing 1	NM_017547.3	HCM
FXN	Frataxin	NM_000144.4	HCM
GAA	Glucosidase, alpha acid	NM_000152.3	HCM
GATA4	GATA-binding protein 4	NM_002052.3	CSD
GATA5	GATA-binding protein 5	NM_080473.4	ASD, AA
GATA6	GATA-binding protein 6	NM_005257.5	ASD, TOF
GATAD1	GATA zinc finger domain containing 1	NM_021167.4	DCM
GJA1	Gap junction protein alpha 1	NM_000165.4	ASD, SIDS
GJA5	Gap junction protein alpha 5	NM_005266.6	Afib

Supp Data Table 1 List of genes (part 3).

Gene	Protein name	RefSeq-Nr.	Disease association
GJD4	Gap junction protein delta 4	NM_153368.2	Afib
GLA	Galactosidase, alpha	NM_000169.2	HCM
GLB1	Galactosidase, beta 1	NM_000404.2	DCM, HCM
GPD1L	Glycerol-3-phosphate dehydrogenase 1–like	NM_015141.3	LQTS
GUSB	Glucuronidase, beta	NM_000181.3	HCM
HCN4	Hyperpolarization-activated cyclic nucleotide–gated potassium channel 4	NM_005477.2	BrS, SSS
HEY2	Hes-related family bHLH transcription factor with YRPW motif 2	NM_012259.2	BrS
HFE	Hemochromatosis	NM_000410.3	DCM
HRAS	Harvey rat sarcoma viral oncogene homolog	NM_005343.2	HCM
HSPB7	Heat shock 27kDa protein family, member 7 (cardiovascular)	NM_014424.4	DCM
ILK	Integrin-linked kinase	NM_001014794.2	DCM
JPH2	Junctophilin 2	NM_020433.4	HCM
JUP	Junction plakoglobin	NM_021991.2	ARVC
KCNA5	Potassium voltage-gated channel, shaker-related subfamily, member 5	NM_002234.3	Afib, LQTS
KCND3	Potassium voltage-gated channel, Shal-related family, member 3	NM_004980.4	BrS
KCNE1	Potassium voltage-gated channel, Isk-related family, member 1	NM_000219.5	LQTS, SQTS
KCNE2	Potassium voltage-gated channel, Isk-related family, member 2	NM_172201.1	Afib, LQTS
KCNE3	Potassium voltage-gated channel, Isk-related family, member 3	NM_005472.4	BrS
KCNE5	Potassium voltage-gated channel, Isk.related family, member 1-like	NM_012282.2	LQTS
KCNH2	Potassium Voltage gated channel, subfamily H, member 2	NM_000238.3	LQTS, SQTS
KCNJ2	Potassium inwardly rectifying channel, subfamily J, member 2	NM_000891.2	CPVT, Afib, LQTS, SQTS
KCNJ5	Potassium inwardly rectifying channel, subfamily J, member 5	NM_000890.3	LQTS
KCNJ8	Potassium inwardly rectifying channel, subfamily J, member 8	NM_004982.3	IVF
KCNQ1	Potassium voltage gated channel, KQT type	NM_000218.2	Afib, LQTS, SQTS
KRAS	Kirsten rat sarcoma viral oncogene homolog	NM_033360.3	Noonan syndrome, HCM
LAMA4	Laminin, alpha 4	NM_001105206.2	DCM
LAMP2	Lysosome associate membrane glycoprotein 2	NM_001122606.1	DCM, HCM
LDB3	LIM binding domain 3 (ZASP)	NM_001080114.1	ARVC, DCM
LMNA	Lamin A/C	NM_170707.2	ARVC, DCM
MAP2K1	Mitogen-activated protein kinase kinase 1	NM_002755.3	HCM
MAP2K2	Mitogen-activated protein kinase kinase 2	NM_030662.3	HCM
MED23	Mediator complex subunit 23	NM_004830.3	IVF
MRPL3	Mitochondrial ribosomal protein L3	NM_007208.3	HCM
MYBPC3	Myosin binding protein C, cardiac	NM_000256.3	DCM, HCM, LVNC
MYH11	Myosin heavy chain 11, smooth muscle	NM_001040114.1	AA

Supp Data Table 1 List of genes (part 4).

Gene	Protein name	RefSeq-Nr.	Disease association
MYH6	Myosin heavy chain, cardiac a	NM_002471.3	ASD, DCM, HCM, SSS
MYH7	Myosin Heavy chain 7, cardiac B	NM_000257.3	DCM, HCM
MYL2	Myosin light chain 2, regulatory, cardiac	NM_000432.3	HCM
MYL3	Myosin light chain 3, ventricular, skeletal, slow	NM_000258.2	HCM
MYLK	Myosin light chain kinase	NM_053025.3	AA, HCM
MYLK2	Myosin light chain kinase 2	NM_033118.3	HCM
MYOM1	Myomesin 1	NM_003803.3	HCM
MYOZ2	Myozenin 2	NM_016599.4	HCM
MYPN	Myopalladin	NM_001256267.1	DCM, HCM
NEBL	Nebulette	NM_006393.2	DCM
NEXN	Nexilin (F actin-binding protein)	NM_144573.3	DCM, HCM
Nkx2-5	NK2 transcription factor-related 5	NM_004387.3	ASD, TOF
NOS1AP	Nitric oxide synthase 1 (neuronal) adaptor protein	NM_014697.2	LQTS
NOTCH1	Notch 1	NM_017617.3	AA
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog	NM_002524.4	Noonan syndrome, HCM
PDLIM3	PDZ and LIM domain 3	NM_014476.5	DCM, HCM
PKP2	Plakophilin2	NM_004572.3	ARVC
PLN	Phosholamban	NM_002667.3	ARVC, DCM, HCM
PRKAG2	Protein kinase, AMP-activated, 2 noncatalytic subunit	NM_016203.3	HCM
PRKG1	Protein kinase, cGMP-dependent type 1	NM_006258.3	TAAD
PSEN1	Presenilin-1	NM_000021.3	DCM
PSEN2	Presenilin-2	NM_000447.2	DCM
PTPN11	Protein tyrosine phosphatase, nonreceptor type 11	NM_002834.3	Noonan syndrome, HCM
RAB3GAP1	RAB3 Gtpase activating protein subunit 1 (catalytic)	NM_001172435.1	SCD
RAF1	v-raf-1 murine leukaemia viral oncogene	NM_002880.3	Noonan syndrome, HCM
RANGRF	RAN guanine nucleotide release factor	NM_016492.4	BrS
RBM20	RNA-binding motif protein 20	NM_001134363.2	DCM
RYR2	Ryanodine receptor	NM_001035.2	CPVT
SCN10A	Sodium channel type 10	NM_001293306.2	BrS, PCCD
SCN1B	Sodium voltage-gated channel beta subunit 1	NM_199037.3	Afib, BrS
SCN2B	Sodium voltage-gated channel beta subunit 2	NM_004588.4	Brs, Afib, IVF
SCN3B	Sodium voltage-gated channel beta subunit 3	NM_018400.3	Brs, Afib, IVF
SCN4B	Sodium voltage-gated channel beta subunit 4	NM_174934.3	Afib, LQTS
SCN5A	Sodium voltage-gated channel alpha subunit 5	NM_001099404.1	BrS, LQTS, PCCD, IVF, SSS, DCM
SCO2	SCO2 cytochrome c oxidase assembly protein	NM_001169109.1	HCM
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	NM_001294332.1	DCM, LVNC

Supp Data Table 1 List of genes (part 5).

Gene	Protein name	RefSeq-Nr.	Disease association
SEMA3A	Sema domain, immunoglobulin domain (lg), short basic domain secreted 3A	NM_006080.2	IVF
SGCD	Sarcoglycan delta (71ystrophin Ass glycoprotein	NM_000337.5	DCM
SHOC2	Soc-2 suppressor of clear homolog	NM_007373.3	Noonan syndrome, HCM
SKI	v-ski avian sarcoma viral oncogene homolog	NM_003036.3	Shprintzen-Goldberg
SLC25A10	Solute carrier family 25 (mitochondrial carrier,dicarboxylate transporter) member 10	NM_001270888.1	ATS, AA
SLC25A3	Solute carrier family 25 (mitochondrial carrier, phosphate carrier) member 3	NM_145305.2	HCM
SLMAP	Sarcolemma associated protein	NM_007159.2	BrS
SMAD3	SMAD family member 3	NM_005902.3	Marfan syndrome, AA
SMAD9	SMAD family member 9	NM_001127217.2	PAH
SNTA1	Syntrophin alpha 1	NM_003098.2	LQTS
SOS1	Son of sevenless homolog 1	NM_005633.3	Noonan syndrome, HCM
SYNE1	Spectrin repeat containing nuclear envelope 1	NM_182961.3	DCM
SYNE2	Spectrin repeat containing nuclear envelope 2	NM_182914.2	ASD, TOF
TAZ	Tafazzin	NM_000116.3	DCM, HCM, LVNC
TBX1	T-box 1	NM_080647.1	DGS / VCFS
TBX5	T-box 5	NM_000192.3	Holt-Oram syndrome
TCAP	Titin cap (telethonin)	NM_003673.3	DCM, HCM
TGFβ2	Transforming growth factor beta 2	NM_001135599.2	Marfan syndrome, AA
TGFβ3	Transforming growth factor B3	NM_003239.2	ARVC
TGFβR1	Transforming growth factor, beta receptor 1	NM_004612.2	Marfan syndrome, MVP, AA
TGFβR2	Transforming growth factor, beta receptor 2	NM_001024847.2	Marfan syndrome, MVP, AA
TMEM43	Transmembrane protein 43	NM_024334.2	ARVC
TMEM70	Transmembrane protein 70	NM_017866.5	HCM
TMPO	Thymopoietin	NM_003276.2	DCM
TNNC1	Troponin C type 1	NM_003280.2	DCM, HCM
TNNI3	Troponin I Type 3 (cardiac)	NM_000363.4	DCM, HCM
TNNT2	Troponin T type 2 (cardiac)	NM_001276345.1	DCM, HCM, LVNC
TP63	Tumor protein p63	NM_003722.4	ARVC
TPM1	Tropomyosin 1(alpha)	NM_000366.5	DCM, HCM, LVNC
TRDN	Triadin	NM_006073.3	CPVT
TRPM4	Transient receptor potential cation channel, subfamily M, member 4	NM_017636.3	PCCD, BrS
TRPM7	Transient receptor potential cation channel, subfamily M, member 7	NM_017672.4	DCM, PCCD
TSFM	Ts translation elongation factor, mitochondrial	NM_001172696.1	HCM
TTN	Titin	NM_001267550.2	DCM, HCM, ARVC
TTR	Transthyretin	NM_000371.3	DCM, HCM

Supp Data Table 1 List of genes (part 6).

Gene	Protein name	RefSeq-Nr.	Disease association
TXNRD2	Thioredoxin reductase 2	NM_006440.4	DCM
VCL	Vinculin	NM_014000.2	DCM, HCM
XK	X-linked Kx blood group (McLeod syndrome)	NM_021083.2	DCM
ZNF365	Zinc finger protein 365	NM_199451.2	SCD

Supp. Table S1 List of genes.

AA aortic aneurysm, *Afib* atrial fibrillation, *ARVC* arrhythmogenic right ventricular cardiomyopathy, *ASD* atrial septal defect, *ATS* arterial tortuosity syndrome, *BrS* Brugada syndrome, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *CSD* cardiac septal defect, *DCM* dilated cardiomyopathy, *DGS* diGeorge syndrome, *HCM* hypertrophic cardiomyopathy, *IVF* idiopathic ventricular fibrillation, *LQTS* long QT syndrome, *LVNC* left ventricular noncompaction cardiomyopathy, *MVP* mitral valve prolapse, *PAH* pulmonary hypertension, *PCCD* progressive cardiac conduction defect, *SCD* sudden cardiac death, *SQTS* short QT syndrome, *SSS* sick sinus syndrome 2, *TAAD* familial thoracic aortic aneurysm and aortic dissection, *TOF* tetralogy of Fallot, *VCFS* velocardiafacial syndrome

2.2 Manuscript II

Post-mortem whole exome analysis in a large sudden infant death syndrome cohort with a focus on cardiovascular and metabolic genetic diseases

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ARTICLE

Post-mortem whole-exome analysis in a large sudden infant death syndrome cohort with a focus on cardiovascular and metabolic genetic diseases

Jacqueline Neubauer*[,1], Maria Rita Lecca[2], Giancarlo Russo[2], Christine Bartsch[3], Argelia Medeiros-Domingo[4], Wolfgang Berger[5,6,7] and Cordula Haas[1]

Sudden infant death syndrome (SIDS) is described as the sudden and unexplained death of an apparently healthy infant younger than one year of age. Genetic studies indicate that up to 35% of SIDS cases might be explained by familial or genetic diseases such as cardiomyopathies, ion channelopathies or metabolic disorders that remained undetected during conventional forensic autopsy procedures. Post-mortem genetic testing by using massive parallel sequencing (MPS) approaches represents an efficient and rapid tool to further investigate unexplained death cases and might help to elucidate pathogenic genetic variants and mechanisms in cases without a conclusive cause of death. In this study, we performed whole-exome sequencing (WES) in 161 European SIDS infants with focus on 192 genes associated with cardiovascular and metabolic diseases. Potentially causative variants were detected in 20% of the SIDS cases. The majority of infants had variants with likely functional effects in genes associated with channelopathies (9%), followed by cardiomyopathies (7%) and metabolic diseases (1%). Although lethal arrhythmia represents the most plausible and likely cause of death, the majority of SIDS cases still remains elusive and might be explained by a multifactorial etiology, triggered by a combination of different genetic and environmental risk factors. As WES is not substantially more expensive than a targeted sequencing approach, it represents an unbiased screening of the exome, which could help to investigate different pathogenic mechanisms within the genetically heterogeneous SIDS cohort. Additionally, re-analysis of the datasets provides the basis to identify new candidate genes in sudden infant death.
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INTRODUCTION

Sudden infant death syndrome (SIDS) is defined as the sudden and unexpected death of an infant younger than one year of age, with the onset of the fatal episode apparently occurring during sleep.[1] The cause of death remains unexplained after a thorough investigation, including performance of a complete autopsy,[2] review of the circumstances of death and the clinical history. Although the incidence rate of SIDS cases drastically decreased in the last years, SIDS is still one of the leading causes of postneonatal infant death in developed countries with a prevalence between 0.1 and 0.8 deaths per 1000 live births.[3] The occurrence of SIDS is described by a triple risk model involving (1) a critical developmental period in the first months after birth, (2) a vulnerable infant and (3) exogenous stress factors.[4] Environmental risk factors such as the prone sleeping position, sharing bed with parents, or smoking exposure during pregnancy are widely accepted stressors to expose a vulnerable infant at risk for cardiorespiratory failure or other homeostatic imbalance.[5] However, the pathophysiological mechanisms responsible for SIDS still remain poorly understood.[6,7] Genetic studies in SIDS cohorts collectively suggest that up to 15% of SIDS cases might be explained by inherited cardiac diseases not detectable during conventional forensic autopsy investigations.[8–10] Ion channelopathies such as Brugada syndrome (BrS), long QT syndrome (LQTS), short QT syndrome or catecholaminergic polymorphic ventricular tachycardia (CPVT), are described as disrupted channel functions causing disturbed ion current flow and lethal cardiac arrhythmias.[11] Cardiomyopathies are characterized by structural abnormalities in the heart, such as hypertrophic cardiomyopathy, dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy (ARVC) and left ventricular non-compaction cardiomyopathy.[12] Additionally, undiagnosed inherited metabolic diseases such as medium-chain-acyl-CoA dehydrogenase (MCAD) deficiency or glucose metabolism deficiency might contribute to the cause of death in another 1% of the SIDS infants.[13]

Post-mortem genetic testing by using massive parallel sequencing (MPS) approaches represents an efficient and rapid strategy to investigate potential disease-causing mechanisms that remained undetected during conventional autopsy and may help to identify the cause of death in some of the SIDS infants and to detect families at risk for further sudden deaths.[14,15] A first MPS-based genetic investigation in 104 genes associated with sudden cardiac death had identified likely pathogenic variants in two cardiomyopathy-associated genes (*PKP2* and *VCL*) in one representative SIDS case.[10] A second MPS-based targeted sequencing study in 47 Danish cases of sudden unexpected death in infancy reported likely causative variants in cardiac disease-

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associated genes in 16 cases (34%), demonstrating the potential of performing a molecular autopsy in sudden death cases.[16] As whole-exome sequencing (WES) is not substantially more expensive than a targeted sequencing approach, this sequencing strategy allows extended data analysis in cases of a negative result within a predefined gene list. Therefore, the aim of this study was to perform a WES analysis in our large SIDS cohort of 161 infant cases with focus on 192 genes associated with cardiovascular and metabolic disorders.

MATERIALS AND METHODS

SIDS study population

Our study population consisted of 161 SIDS cases collected between 1985 and 2014 at the Zurich Institute of Forensic Medicine, Zurich, Switzerland. Most of the SIDS cases were examined by the same forensic pathologist, ensuring a high level of uniformity in autopsy procedures and case reporting. The classification of SIDS cases has always been performed according to the generally accepted international definitions of SIDS, including a complete autopsy, review of the circumstances of death, and examination of the clinical history.[1]

Forty-one infants were determined as genuine SIDS cases belonging to SIDS category I, including infants with normal clinical history, normal growth and development, no similar deaths among siblings and found in a safe sleeping environment with no evidence of accidental death. The remaining 120 infants were classified into SIDS category II due to slight infections before death, preterm birth or other deviations to category I requirements. The median age of the 161 SIDS infants was 15.03 ± 8.3 weeks (range 0.6–48.1 weeks) and 60.2% were boys (97 males/64 females). All of the SIDS infants were Europeans, most of them Swiss. A targeted MPS approach (HID-Ion AmpliSeq Ancestry Panel, Thermo Fisher, Rotkreuz, Switzerland) using the Ion Torrent PGM platform (Thermo Fisher) was applied to verify the geographical origin of SIDS cases where no information on the family origin was available. Eleven SIDS cases were excluded from our original cohort of 172 individuals, because of non-European ethnicities. Even small amounts of population admixture can shift the results toward an association, and therefore, it is important to have a well-defined study population in regard to ethnic and geographic background.[17] Additional epidemiological data of the SIDS cohort are illustrated in Supplementary Table 1.

Ethical approval for this study was provided by the local ethics committee (KEK-ZH-Nr. 2013–0086), and the study was conducted in full conformance with Swiss laws and regulations. Family members were not available for co-segregation analysis.

DNA extraction and quantification

Genomic DNA of the SIDS infants was obtained from tissues stored in alcohol or from alcohol-fixed and paraffin-embedded tissue blocks.[18] In most of the cases kidney or tongue was used (otherwise heart, muscle or brain) because of reported good post-mortem DNA stability in these tissues.[19] DNA extractions were performed using the QIAamp DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. All DNA quantities were determined with a Qubit 1.0 fluorometric quantification device (Thermo Fisher).

Exome sequencing and bioinformatics

DNA library preparation and exome capture were performed with the SureSelect target enrichment and SureSelect All Exon V5+UTR's kits (Agilent Technologies AG, Basel, Switzerland), using the protocol for 200 ng input amount of genomic DNA. Sequencing as well as sequence alignment and variant calling were performed at the Functional Genomics Center Zurich, Switzerland. Sequencing was done on the Illumina HiSeq2500 platform (Illumina, San Diego, USA), generating 2×100 bp paired-end reads. Sequences were aligned to the reference genome (GRCh37/hg19) using BWA.[20] MEM algorithm with default setting and quality control of the exome coverage was performed with Bioconductor package TecQC.[21] A filter was set so that a sample was required to have at least 80% of exome covered at $\geq 20\times$ read depth. Variants discovery was performed by means of GATK,[22] following the GATK best practices workflow.[23] In particular, groups were reassigned using PicardTools,[24] duplicate reads were removed using Samtools[25] and local realignment, variants discovery and filtering (minimum $20\times$ coverage, minimum 20% alternate allele frequency) was done with GATK walker and the dbSNP database.[26]

Data analysis

Within our WES results, we focused on a gene panel of 192 genes associated with cardiovascular or metabolic disease-associated genes (Supplementary Table 2). Variants with $<50\times$ bidirectional coverage and/or an alternate allele frequency ratio <0.4 were additionally confirmed by standard Sanger sequencing methods. Annotation of the variants was performed with the Software Alamut Batch version 1.4.2 (Interactive Biosoftware, Rouen, France). Output results were reported in an Excel-sheet for data analysis. Variants in *TTN* were not further evaluated due to reported difficulties in sequencing and variant interpretation.[27] We adapted our previously published filter strategy[28] as follows: (1) a global minor allele frequency value (MAF) of ≤ 0.01 based on NCBI dbSNP,[26] (2) focus on exonic and splice site variants and (3) the exclusion of synonymous variants (Supplementary Figure 1). In addition, Human Gene Mutation Database (HGMD, Qiagen)[29] was consulted to check already reported variants in the literature. Alamut Visual Version 2.7.1 (Interactive Biosoftware) was used to visualize coverage of variants and to review conservation of the variants across a variety of species. Pathogenicity of variants was assigned according to an adapted scoring scheme originally described by van Spaendonck-Zwarts *et al.*[30] and Hertz *et al.*[31] Our scoring scheme was based on the assessment of variant types (null-variants, splice site variants, missense variants), *in silico* protein predictions, and MAF in three European control populations namely Exome Sequencing Project (ESP), 1000 Genomes Project and Exome Aggregation consortium (ExAC) database (Table 1).[26,32–39] A small proportion of the 60 706 individuals in the ExAC database is originally coming from smaller databases possibly leading to an overlap of the European individuals.[40] ESP is not well-powered to filter at 0.1% allele frequency without removing many genuinely rare variants, however the majority of ESP European singletons are not seen a second time in ExAC. Therefore, we still used the European MAF of all three databases. Based on the scoring scheme, variants were classified into five separate subcategories, designated as variants of unknown significance VUS0-4 (Table 2). Co-segregation and functional analyses would have been required to classify a variant as pathogenic;[30] therefore, the highest score a variant in our study could get was VUS4. DNA variants were numbered according to reference sequences using HGVS nomenclature (http://varnomen.hgvs.org). Variants in subcategories VUS4/VUS3 have been submitted to the Leiden Open Variation Database (Individual IDs: 00064759 to 00064769/00065124 to 00065157) (http://databases.lovd.nl/shared/diseases/02087).

Variant confirmation

Potential disease-causing variants not reported in the mentioned databases were confirmed by Sanger sequencing. Additionally, allele frequencies were checked in an in-house exome database of 118 European patients with eye diseases.

RESULTS

Whole-exome sequencing and data analysis was successfully completed for 155 SIDS cases. DNA library preparation failed for six SIDS samples due to low DNA quantities or poor DNA qualities resulting from decayed post-mortem tissues and/or the fixation method.

Overall, 72.3% of the bases had a coverage of ≥ 20 reads and the average on-target coverage was 90.2% at ≥ 20 reads. The average depth within our gene panel was 100.64 ± 34.25 (Supplementary Table 3). By focusing on 192 genes of interest, an average of 1960 ± 467 variants per case were obtained for further data analysis. After the filtering steps, an average of 14.6 ± 9.8 variants per sample were manually checked with the Alamut Visual v2.7 software and evaluated according to our scoring scheme. Sanger sequencing confirmed all VUS4/VUS3 variants with $<50\times$ coverage and/or not reported in the databases (Supplementary Table 4). The majority of these variants were missense

Table 1 Scoring scheme for variant evaluation (adapted from Hertz *et al.*[31])

Parameter	*Score*
Coding effect	
Nonsense	24
Frameshift	24
Splice sites	
±1/±5	24
±2/±3	12
All others	0
Missense	0
In silico protein prediction	
AGVGD	
C65	4
C55	3.2
C35	2.4
C25	1.6
C15	0.8
C0	0
NA	0
Grantham distance	
>140	4
70–140	2
<70	0
NA	0
SIFT	
Deleterious	4
Good	0
NA	0
MAPP	
Bad	4
Good	0
NA	0
Polyphen2	
Probably damaging	4
Possibly damaging	2
Benign	0
MutationTaster	
Disease causing	4
Polymorphism	0
NA	0
MAF	
ESP EAMAF	
≤0.001	8
0.001<freq≤0.002	5.3
0.002<freq≤0.01	2.6
>0.01	0
NA	0
ExAC EURNFMAF	
≤0.001	8
0.001<freq≤0.002	5.3
0.002<freq≤0.01	2.6
>0.01	0
NA	0
1000 Genomes Project EURMAF	
≤0.001	8
0.001<freq≤0.002	5.3
0.002<freq≤0.01	2.6
>0.01	0
NA	0

Abbreviations: AGVGD, align Grantham variation and Grantham deviation[32]; ESP EAMAF, MAF in European American population in NHLBI GO Exome Sequencing Project[37]; ExAC EURNFMAF, MAF in European (non-Finnish) population in exome aggregation consortium[39]; MAF, minor allele frequency; MAPP, multivariate analysis of protein polymorphism prediction[34]; NA, not available; Polyphen2, polymorphism phenotyping v2 (ref. 35); SIFT, sorting intolerant from tolerant prediction[33]; MutationTaster[36]; 1000 Genomes Project EURMAF, MAF in European population in 1000 Genomes Project.[38]

Table 2 Subcategories of variants based on scoring scheme

Percentage (%)	*Score*	*Subclass*	
≥90	43.2–48.0	VUS4	Probably pathogenic
80–89	38.4–43.1	VUS3	Likely pathogenic
70–79	33.6–38.3	VUS2	Unclear
60–69	28.8–33.5	VUS1	Unlikely pathogenic
<59	≤28.7	VUS0	Not pathogenic (neutral variant or weak modifier)

Abbreviation: VUS, variant of unknown significance.

variants (97.1%) followed by splice site variants (1.5%), nonsense variants (0.9%), and frameshift variants (0.6%). Based on our scoring scheme, 11 variants (1.3%) were classified into sub-category VUS4 and 24 variants (2.7%) into VUS3 indicating variants with probably pathogenic effects.

Thirty-one (20%) out of the 155 SIDS cases had at least one variant with likely pathogenic functional effects (Table 3). Four of these cases had two likely causative variants. Details of the variants are available in Supplementary Table 4. Of the 31 SIDS cases, 17 (54.8%) were males and the median age of death was 4 months (range 1–9 months). Most of the variants were detected in SIDS category II infant cases (67.7%).

Among the 31 SIDS infants with likely causative variants, 14 infants (9%) carried putative pathogenic variants in genes associated with ion channelopathies and 11 SIDS infants (7%) had disease-causing variants in genes associated with cardiomyopathies (Figure 1). Additional four SIDS infants (2.5%) had variants in genes associated with mitral valve prolapse, aortic valve disease, Marfan syndrome or Ehlers-Danlos syndrome. Variants in genes associated with metabolic diseases were found in two SIDS cases (1%), in glycogen storage disease and systemic primary carnitine deficiency.

Most of the variants were detected in genes associated with BrS (2.5%), followed by dilated cardiomyopathy (2.1%), hypertrophic cardiomyopathy (1.4%), LQTS (1.4%), ARVC (1.0%) and CPTV (1.0%).

DISCUSSION

High-throughput sequencing provides a comprehensive and time-efficient sequencing strategy to identify rare DNA sequence variants in the genome/exome of patients with complex disorders or to discover underlying genetic causes in large heterogeneous study populations as for example in SIDS cases.

Starting with tissue collection of SIDS infants already in the early 1980s, we have a valuable and well-defined SIDS cohort of 161 infant cases at our institute. Although alcohol-fixed and paraffin-embedded tissue blocks do not provide optimal DNA qualities and quantities, exome sequencing was successfully completed for 155 out of 161 SIDS cases. By focusing on a gene list of 192 genes associated with cardiovascular or metabolic diseases, we identified potentially disease-causing variants in 20% of the 155 SIDS cases. The majority of these cases had variants in genes associated with channelopathies (9%) and cardiomyopathies (7%).

The main cardiac genes reported in other SIDS studies are *CAV3*, *GJA1*, *GPD1-L*, *KCNE2*, *KCNJ8*, *KCNQ1*, *KCNH2*, *MYBPC3, RYR2*, *SCN5A* and *TNNI3*.[6] We detected potentially causative variants in two of our SIDS cases in *SCN5A* p.(Arg1897Trp) and *RYR2* (c.2907-1G>C). *SCN5A* is primarily expressed in the cell membrane of cardiac tissue where it encodes sodium-gated channels. Although *SCN5A* p.(Arg1897Trp) has been reported in patients with LQTS and

Table 3 Variants with likely disease-causing effects in the SIDS cohort

Case	Sex	Age (mo)	rs-Nr.	Gene	HGVS genomic RefSeq-Nr.	HGVS RefSeq-Nr.	Coding Effect	c.DNA	Protein change	dbSNP ALLMAF	ESP EAMAF	ExAC EURNFMAF	1000 Genomes EURMAF	in-house database[a]	AGVGD	SIFT	MAPP	MutationTaster	Grantham distance	Polyphen2	Disease association	Subclass based on scoring scheme
SIDS001	F	9	rs17848485	*CPT2*	NG_008035.1	NM_000098.2	missense	c.1634A>C	p.(Glu545Ala)	0.0010	0.0000	0.0000	0.0030	NA	C65	deleterious	bad	disease-causing	107	probably damaging	SPCD	VUS3
SIDS004	M	7	rs199583774	*PKP2*	NG_009000.1	NM_004572.3	missense	c.2083C>T	p.(Arg695Cys)	0.0000	0.0000	0.0001	0.0010	NA	C25	deleterious	bad	disease-causing	180	probably damaging	ARVC	VUS4
SIDS005	M	4	rs142890619	*COL5A1*	NG_008030.1	NM_000093.4	missense	c.5986>A	p.(Asp2004Asn)	0.0000	0.0010	0.0000	0.0010	NA	C0	deleterious	bad	disease-causing	23	probably damaging	EDS	VUS3
SIDS007	F	8	rs143641133	*DOLK*	NG_017009.1	NM_014908.3	missense	c.1324G>A	p.(Ala442Thr)	0.0000	0.0000	0.0000	0.0010	NA	C0	deleterious	bad	disease-causing	58	probably damaging	DCM	VUS3
SIDS010	F	3	rs200473206	*DSP*	NG_008803.1	NM_004415.3	splice site	c.273+5G>A	p.(?)	0.0000	0.0005	0.0005	NA	NA	NA	NA	NA	NA	NA	NA	ARVC	VUS3
SIDS011	M	2	rs71352737	*TRPM4*	NG_027551.1	NM_017636.3	nonsense	c.1575G>A	p.(Trp525*)	0.0010	0.0011	0.0018	0.0010	NA	NA	NA	NA	disease-causing	NA	NA	BrS	VUS4
SIDS015	M	1	rs143788120	*MMP13*	NG_029207.1	NM_002208.3	missense	c.862T>C	p.(Ser288Pro)	0.0000	0.0000	0.0006	0.0030	NA	C65	deleterious	bad	disease-causing	74	probably damaging	HCM	VUS3
SIDS021	M	4	~	*TSFM*	NG_016977.1	NM_001172696.1	frameshift	c.971_972del	p.(Gln324Argfs*11)	NA	0.0000	NA	0.0000	0.0000	NA	NA	NA	NA	NA	NA	DCM	VUS3
SIDS024	M	2	rs140470576	*AKAP9*	NG_011623.1	NM_005751.4	missense	c.10118C>A	p.(Ser3373Tyr)	0.0000	0.0020	0.0000	0.0000	NA	C65	deleterious	bad	disease-causing	144	probably damaging	LQTS	VUS4
SIDS031	F	4	rs141131355	*MYLK*	NG_029111.1	NM_053025.3	missense	c.3446>A	p.(Arg1153His)	0.0000	0.0002	0.0001	0.0010	NA	C25	deleterious	bad	disease-causing	29	possibly damaging	HCM	VUS3
SIDS037	F	4	rs139965373	*AKAP9*	NG_011623.1	NM_005751.4	missense	c.35806>A	p.(Ile11941Thr)	0.0010	0.0000	0.0000	0.0000	NA	C55	deleterious	bad	disease-causing	58	probably damaging	LQTS	VUS4
SIDS041	F	4	rs185947256	*ACG*	NG_012865.1	NM_000038.2	missense	c.3290G>A	p.(Arg1097His)	0.0000	0.0000	0.0000	0.0000	NA	C0	deleterious	bad	disease-causing	29	probably damaging	GSD	VUS3
SIDS045	M	5	rs369958881	*SCN1B*	NG_013361.1	NM_199037.3	missense	c.482G>T	p.(Arg161Leu)	0.0000	0.0000	0.0001	0.0000	NA	C0	deleterious	bad	disease-causing	102	probably damaging	BrS	VUS4
SIDS050	M	2	rs199732064	*TRPM7*	NG_027551.1	NM_017672.4	missense	c.4006C>T	p.(Pro1336Ser)	0.0000	0.0000	0.0005	0.0010	NA	C0	deleterious	bad	disease-causing	74	possibly damaging	DCM	VUS3
SIDS061	M	4	rs142217269	*SCN10A*	NG_031891.1	NM_006514.3	missense	c.4568G>A	p.(Cys1523Tyr)	0.0010	0.0027	0.0018	0.0010	NA	C65	deleterious	bad	disease-causing	194	probably damaging	BrS	VUS3
			rs143767864	*DCHS1*	NG_033858.1	NM_003737.3	missense	c.7046>A	p.(Arg2349Gln)	0.0000	0.0000	0.0000	0.0010	NA	C0	deleterious	bad	disease-causing	43	probably damaging	MVP	VUS3
SIDS069	M	3	rs200893930	*NOTCH1*	NG_007458.1	NM_017617.4	missense	c.7645C>T	p.(Arg2549Cys)	0.0000	0.0001	0.0000	0.0000	NA	C25	deleterious	bad	disease-causing	180	possibly damaging	AOVD	VUS4
SIDS073	M	3	rs200473226	*DSP*	NG_008803.1	NM_004415.2	splice site	c.273+5G>A	p.(?)	0.0000	0.0005	0.0005	NA	NA	NA	NA	NA	NA	NA	NA	ARVC	VUS3
SIDS075	F	3	rs121434557	*GJA5*	NG_009369.2	NM_005266.6	missense	c.286G>T	p.(Ala96Ser)	0.0000	0.0000	0.0001	0.0010	NA	C65	deleterious	NA	disease-causing	99	probably damaging	Afib	VUS3
			rs149272010	*SYNE1*	NG_012855.1	NM_182961.3	missense	c.19534G>T	p.(Ala6512Thr)	NA	0.0000	0.0000	0.0000	NA	C55	deleterious	bad	NA	58	probably damaging	EDMD	VUS3
SIDS087	M	4	~	*MYLK*	NG_029111.1	NM_053025.3	missense	c.1490C>T	p.(Ser497Phe)	NA	0.0000	NA	NA	0.0000	C65	deleterious	bad	disease-causing	155	probably damaging	HCM	VUS3
SIDS089	F	5	rs199800922	*MYH11*	NG_009299	NM_001040114.1	missense	c.3624C>G	p.(His1208Gln)	0.0000	0.0001	0.0000	0.0010	NA	C15	deleterious	bad	disease-causing	24	possibly damaging	HCM	VUS3
SIDS091	M	2	rs200899037	*CASQ2*	NG_008802.1	NM_001232.3	missense	c.1052A>G	p.(Asp351Gly)	0.0000	0.0000	0.0000	0.0010	NA	C0	deleterious	bad	disease-causing	94	probably damaging	CPVT	VUS3
SIDS096	F	2	rs71352737	*TRPM4*	NG_027551.1	NM_017636.3	nonsense	c.1575G>A	p.(Trp525*)	0.0010	0.0011	0.0018	0.0010	NA	NA	NA	NA	disease-causing	NA	NA	BrS	VUS4
SIDS100	F	4	rs373347787	*SCN10A*	NG_031891.2	NM_006514.3	missense	c.4780C>T	p.(Arg1594Cys)	0.0000	0.0000	0.0000	NA	NA	C65	deleterious	bad	disease-causing	180	probably damaging	BrS	VUS3
SIDS101	M	6	rs200899037	*CASQ2*	NG_008802.1	NM_001232.3	missense	c.1052A>G	p.(Asp351Gly)	0.0000	0.0000	0.0000	0.0010	NA	C0	deleterious	bad	disease-causing	94	probably damaging	CPVT	VUS3
SIDS119	F	1	~	*TRPM4*	NG_027551.1	NM_017636.3	frameshift	c.2476dup	p.(Ala836Glyfs*13)	NA	0.0000	NA	NA	0.0000	NA	NA	NA	NA	NA	NA	BrS	VUS3
			rs140148322	*FHL2*	NG_008844.2	NM_001450.3	missense	c.337C>T	p.(Arg113Cys)	0.0000	0.0005	0.0005	NA	NA	C65	deleterious	bad	disease-causing	180	probably damaging	DCM	VUS3
SIDS142	M	3	rs45465995	*SCN5A*	NG_008934.1	NM_001099404.1	missense	c.5689C>T	p.(Arg1897Trp)	0.0020	0.0004	0.0000	0.0020	NA	C65	deleterious	bad	disease-causing	101	probably damaging	BrS	VUS4
SIDS156	F	2	rs139170018	*SYNE1*	NG_012855.1	NM_182961.3	missense	c.15337G>A	p.(Val5113Ile)	0.0000	0.0012	0.0010	0.0000	NA	C25	deleterious	bad	disease-causing	29	probably damaging	EDMD	VUS3
SIDS161	M	1	rs377061868	*TPM1*	NG_007557.1	NM_000366.5	splice site	c.375-5T>C	p.(?)	0.0000	0.0005	0.0004	NA	NA	NA	NA	NA	NA	NA	NA	DCM	VUS3
SIDS172	M	8	rs111843122	*RYR2*	NG_008799.2	NM_001035.2	splice site	c.2907-1G>C	p.(?)	0.0000	0.0000	0.0000	0.0000	NA	NA	NA	NA	NA	NA	NA	CPVT	VUS3
SIDS194	F	5	rs72544141	*ANK2*	NG_009006.2	NM_001148.4	missense	c.4373A>G	p.(Glu1458Gly)	0.0010	0.0002	0.0006	0.0010	NA	C65	deleterious	bad	disease-causing	98	possibly damaging	LQTS	VUS4
SIDS223	F	2	rs148323523	*DCHS1*	NG_033858.1	NM_003737.3	missense	c.6467T>A	p.(Val2156Glu)	0.0000	0.0006	0.0002	0.0000	NA	C65	deleterious	bad	disease-causing	121	possibly damaging	MVP	VUS4

Abbreviations: Afib, atrial fibrillation; AGVGD, align Grantham variation and Grantham deviation; ALLMAF, minor allele frequency in all populations based on NCBI dbSNP; AOVD, aortic valve disease; ARVC, arrhythmogenic right ventricular cardiomyopathy; BrS, Brugada syndrome; CPVT, catecholaminergic polymorphic ventricular tachycardia; DCM, dilated cardiomyopathy; EDMD, Emery-dreifuss muscular dystrophy; EDS, Ehlers-Danlos syndrome; ESP EAMAF, MAF in European American population in NHLBI GO Exome Sequencing Project; ExAC EURNFMAF, MAF in European (non-Finnish) population in exome aggregation consortium; F, female; GSD, glycogen storage disease; HCM, hypertrophic cardiomyopathy; LQTS, long QT syndrome; M, male; MAF, minor allele frequency; MAPP, multivariate analysis of protein polymorphism prediction; MVP, mitral valve prolapse; MS, marfan syndrome; NA, not available; SIFT, sorting intolerant from tolerant prediction; SPCD, systemic primary carnitine deficiency; 1000 Genomes Project EURMAF, MAF in European population in 1000 Genomes Project.
~ No dbSNP rs-no. available.
[a]Based on 118 in-house control exoms.

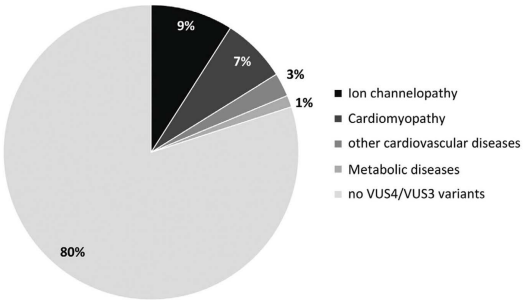

Figure 1 Percentage of SIDS infants with likely causative variants in genes associated with cardiomyopathies, ion channelopathies, other cardiovascular diseases, and metabolic diseases. VUS, variant of unknown significance.

atrial fibrillation, functional studies have indicated no effect on QTc intervals, syncope propensity, and overall mortality assuming that this variant is less likely associated with a dominant monogenic form of the disease.[41] *RYR2* encodes a ryanodine receptor found in cardiac sarcoplasmic reticulum and causative variants are described in stress-induced CPTV and ARVC.[42] *RYR2* (c.2907-1G>C) is located at the 5′-end of exon 26 and causes an altered acceptor site, however the variant was not described in ARVC or CPTV patients so far. Further variants with less likely functional effects were detected in *KCNE2*, *CAV3*, *RYR2* and *MYBPC3* (Supplementary Table 4), but no variants in *GJA1*, *GPD1-L*, *KCNH2*, *KCNJ8* and *TNNI3*.

Additional variants with likely functional effects were found in genes related to different cardiac diseases or sudden death such as *ANK2* p.(Glu148Gly), *ACTN2* p.(Glu891*), *DSP* (c.273+5G>A), *TRPM4* p.(Ala83Glfs*13), *TRPM4* p.(Trp525*), and *TSFM* p.(Gln324Argfs*11). Interestingly, *ANK2* p.(Glu148Gly) and *TRMP4* p.(Trp525*) were already described in a Danish SIDS cohort.[16] *ANK2* encodes ankyrin-B, which has an essential role in the localization and membrane stabilization of ion transporters and ion channels in cardiomyocytes.[43] The same variant was first reported in a large French family with LQTS including sinus node dysfunction and episodes of atrial fibrillation and one individual who suffered sudden death,[44] but *ANK2* p.(Glu148Gly) was also detected in eight Danish control individuals with normal mean QTc interval.[41] *TRPM4* belongs to the melastatin-related transient receptor channel family and encodes calcium-permeable cation channels localized predominantly in the plasma membrane.[45] Variants in *TRPM4* were described in patients with progressive familial heart block and BrS. Both alterations represent interesting candidate variants involved in the sudden death event of SIDS cases.

The most investigated gene with regard to metabolic diseases in SIDS is *ACADM*, which catalyzes the first step in the beta-oxidation of fatty acids.[13] The most prevalent variation causing MCAD deficiency is *ACADM* p.(Lys329Glu), which is present in 80% of individuals who clinically are diagnosed with MCAD.[7] The only variant detected in our SIDS cohort with regard to *ACADM* was *ACADM* p.(Arg53Cys) (Supplementary Table 4). Although this variant has been reported in one MCAD-patient in combination with the most common *ACADM* p.(Lys329Glu) pathogenic variant,[46] our scoring scheme predicts little functional effect for this variant.

Altered ion channel functions causing lethal arrhythmias may represent the most plausible and comprehensible cause in infant death cases.[10] Many channelopathies are characterized by incomplete penetrance and variable expressivity where sudden cardiac death is often the first manifestation of the disease.[47] In contrast, cardiomyopathies are mainly caused by variants in genes encoding desmosomal cell adhesion proteins or in sarcomeric proteins involved in heart contraction inducing structural heart abnormalities. However, a growing number of studies have established links between desmosomes and components of cardiac electrical machinery.[48] Consequently, variants in cardiomyopathy-associated genes may contribute more generally to cardiac diseases and might be involved in the cause of death in some of the SIDS infants even in absence of morphological abnormalities in the heart.

Today, exome sequencing is not substantially more expensive compared to targeted gene panels, but represents a more efficient and comprehensive sequencing method to investigate sudden unexplained death cases in absence of a specific phenotype. To our knowledge, this is the first WES study in a large SIDS cohort. Although we exclusively report the findings within predefined genes of interest, one major advantage of exome sequencing is the alternative of extended data analysis in cases without any results providing a chance to identify new candidate genes in SIDS. The underlying cause of death in the majority of SIDS cases still remains elusive and might be explained by a multifactorial etiology due to a combination of different genetic and environmental risk factors. Therefore, further analyses could focus on SIDS-related predisposing genetic factors in genes involved in early brain development, respiratory regulation, nicotine response, immune system, metabolic and energy production, thermoregulation and mitochondrial activity.[6,7]

The main current challenge in exome sequencing studies is the clinical interpretation of genetic variants identified. The categorization of variants in our study was based on a stringent scoring scheme involving different population-specific databases and *in silico* protein prediction tools. Nevertheless, the different VUS categories included single variants with a higher allele frequency than expected for specific cardiovascular diseases meaning that a part of the here reported VUS might not be severe enough to cause death in infancy; still some of them could act as predisposing risk factors whereas others might be reclassified as benign based on prospective findings. Therefore, further assessments such as functional studies are required and strongly recommended for an evidence-based classification of the pathogenicity.[49] Recently, mutations in genes previously associated with SIDS were identified in exome data from population studies indicating that many variants might have some pathological influence, but are most likely not the exclusive genetic cause of SIDS.[50] Therefore, caution is needed when translating such exome sequencing results from research to diagnostic applications. Genetic counseling of first-degree relatives should be based on a multidisciplinary approach, involving forensic pathologists, geneticists and cardiologists, to inform the family in case of positive genetic findings and to discuss further steps regarding genetic testing of family members and/or to monitor the affected person.[2]

A limitation of this study is the lack of functional assays in order to verify the potentially pathogenic role of detected variants, in particular amino acid substitutions. Also, family members were not available for co-segregation analyses due to the sample anonymity required by the ethical committees. This would be necessary to determine the mode of inheritance, to classify variants into the pathogenic category,[30] and to identify other genetic carriers at risk for sudden cardiac death. Our case reports only included clinical records on sudden death cases in siblings but not in other family members, which would be an important point to consider. Finally, exome sequencing data reveal lower sequencing coverage compared to targeted gene panels

potentially leading to a loss of important low-coverage variants and more false negative/positive calls.

Additional MPS studies combined with functional assessment in large SIDS cohorts are inevitable to better understand the etiology of SIDS and to identify additional pathophysiologic mechanisms involved in this tragic death event.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary information

Neubauer, et al. Post-mortem whole exome analysis in a large sudden infant death syndrome cohort with a focus on cardiovascular and metabolic genetic diseases

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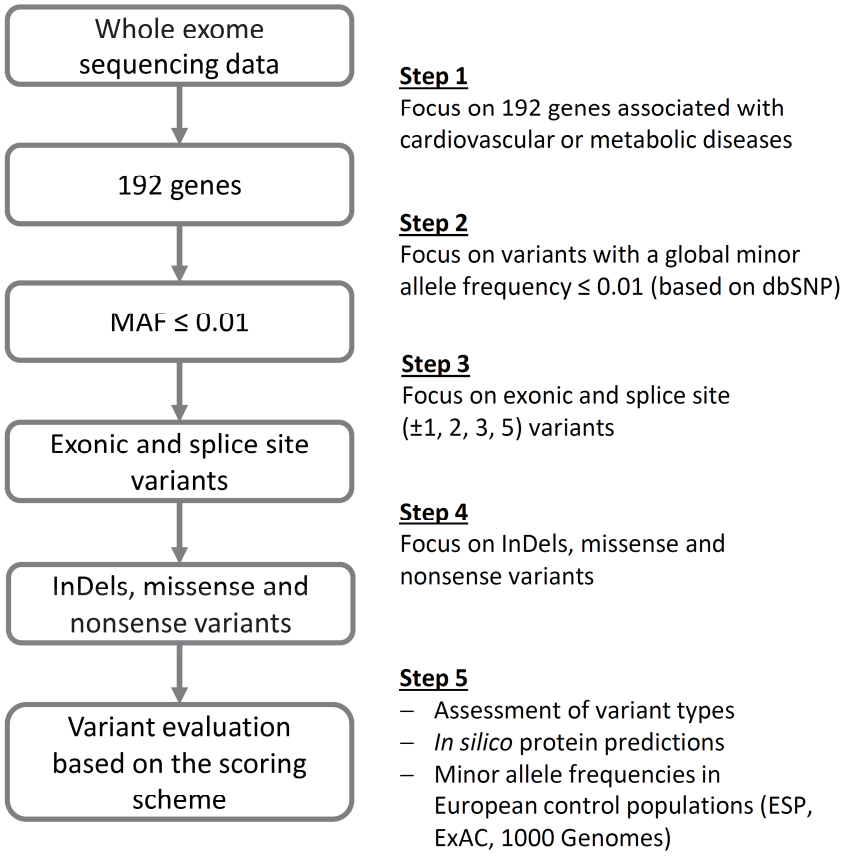

Supp. Data Figure S1 Filtering flow chart (adapted from Neubauer, *et al.*[28])

Whole exome sequencing data were filtered for 192 genes associated with cardiovascular and metabolic diseases. The filtering steps focused on a global MAF less than 0.01 (step 2), exonic and splice site variants (step 3), InDels, missense and nonsense variants (step 4), and variant evaluation based on the scoring scheme (step 5).

InDels: insertions and deletions, MAF: minor allele frequency

Supp. Data Table S1 Epidemiological data of the SIDS cohort.

	SIDS I	SIDS II	All SIDS Cases
Nr. of individuals	41	120	161
Gender			
male	24	73	97
female	17	47	64
Age in weeks (range)			
overall	17.1 ± 10.2 *(4.0 - 34.4)*	17.2 ± 10.6 *(0.6 - 48.1)*	17.2 ± 10.4 *(0.6 - 48.1)*
male	17.1 ± 10.0 *(4.0 - 34.4)*	17.1 ± 10.4 *(2.5 - 43.0)*	17.3 ± 10.6 *(4.0 - 43.0)*
female	17.3 ± 10.5 *(4.8 - 22.3)*	17.0 ± 10.4 *(0.6 - 48.1)*	*17.0 ± 10.4(0.6 - 48.1)*
Sleeping position at death			
prone-sleeping position	12 *(29.3%)*	48 *(40.0%)*	60 *(37.2%)*
supine-sleeping position	5 *(12.2%)*	23 *(19.1%)*	28 *(16.7%)*
unknown	24 *(58.5%)*	49 *(40.9%)*	73 *(45.1%)*
Sharing bed with parents at death			
yes	5 *(12.2%)*	18 *(15.0%)*	23 *(14.3%)*
no	29 *(70.7%)*	83 *(69.2%)*	112 *(69.6%)*
unknown	7 *(17.1%)*	19 *(15.8%)*	26 *(16.1%)*
Exposed to smoking			
yes	8 *(19.5%)*	30 *(25.0%)*	38 *(23.6%)*
no	3 *(7.3%)*	25 *(20.8%)*	28 *(17.4%)*
unknown	30 *(73.2%)*	65 *(54.2%)*	95 *(59.0%)*
Preterm birth			
yes	0 *(0%)*	17 *(14.2%)*	17 *(10.5%)*
no	28 *(68.3%)*	71 *(59.2%)*	99 *(61.5%)*
unknown	13 *(31.7%)*	32 *(26.6%)*	45 *(28.0%)*
Vaccination			
yes	11 *(26.8%)*	37 *(30.8%)*	48 *(29.8%)*
no	3 (7.3%)	11 *(9.2%)*	14 *(8.7%)*
unknown	27 *(65.9%)*	72 *(60.0%)*	99 *(61.5%)*

Supp. Data Table S2 Investigated genes and associated disease (part 1).

Disease	Gene	CHR	Protein name	RefSeq-Nr.	Other associated diseases
Cardiomyopathies					
ARVC	*CTNNA3*	10	Catenin (cadherin-associated protein), alpha 3	NM_001127384.2	
	DSC2	18	Desmocollin 2	NM_024422.4	
	DSG2	18	Desmoglein 2	NM_001943.3	DCM
	DSP	6	Desmoplakin	NM_004415.2	DCM
	JUP	17	Junction plakoglobin	NM_021991.2	
	PKP2	12	Plakophilin2	NM_004572.3	BrS
	TGFβ3	14	Transforming growth factor B3	NM_003239.2	LDS, TAAD
	TMEM43	3	Transmembrane protein 43	NM_024334.2	EDMD
DCM	*ABCC9*	12	ATP-binding cassette, subfamily C (CFTR/MRP), member 9	NM_005691.3	Afib, BrS
	ACTN2	1	Actinin, alpha 2	NM_001103.3	HCM
	BAG3	10	Bcl2-associated athanogene 3	NM_004281.3	Muscular dystrophy
	CTF1	16	Cardiotrophin 1	NM_001330.3	Hypertensive heart disease
	CRYAB	11	Crystallin, a B	NM_001289807.1	Fatal infantile hypertonic myofibrillar myopathy
	DES	2	Desmin	NM_001927.3	Limb-girdle muscular dystrophy
	DMD	X	Dystrophin, muscular dystrophy	NM_004006.2	Duchenne muscular dystrophy, Becker muscular dystrophy
	DNAJC19	3	DNAJ (Hsp40) homolog, subfamily C, member 19	NM_145261.3	3-methylglutaconic aciduria
	DOLK	9	Dolichol kinase	NM_014908.3	Congenital disorder of glycosylation
	EYA4	6	Eyes absent homolog 4 (Drosophila)	NM_172105.3	Non-syndromic hearing loss and deafness
	FHL2	2	Four and a half LIM domains 2	NM_001450.3	Hemophagocytic lymphohistiocytosis
	FKTN	9	Fukuyama-type congenital muscular dystrophy (fukutin)1	NM_006731.2	Muscular dystrophy-dystroglycanopathy
	GAA	17	Glucosidase, alpha acid	NM_000152.3	Congenital disorder of glycosylation
	GATAD1	7	GATA zinc finger domain containing 1	NM_021167.4	
	ILK	11	Integrin-linked kinase	NM_001014794.2	
	LAMA4	6	Laminin, alpha 4	NM_001105206.2	
	LDB3	10	LIM binding domain 3 (ZASP)	NM_001080114.1	HCM, LVNC
	LMNA	1	Lamin A/C	NM_170707.2	ARVC, EDMD
	MYPN	10	Myopalladin	NM_001256267.1	HCM
	NEBL	10	Nebulette	NM_006393.2	
	NEXN	1	Nexilin (F actin-binding protein)	NM_144573.3	HCM, ASD, LVNC
	PLN	6	Phospholamban	NM_002667.3	HCM
	PSEN1	14	Presenilin-1	NM_000021.3	
	PSEN2	1	Presenilin-2	NM_000447.2	
	RBM20	10	RNA-binding motif protein 20	NM_001134363.2	
	RAF1	3	Raf-1 proto-oncogene, serine/threonine kinase	NM_002880.3	Noonan syndrome
	SDHA	5	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	NM_001294332.1	Mitochondrial complex deficiency
	SGCD	5	Sarcoglycan delta (dystrophin Ass glycoprotein	NM_000337.5	Limb-girdle muscular dystrophy
	TAZ	X	Tafazzin	NM_000116.3	LVNC, BTHS
	TCAP	17	Titin cap (telethonin)	NM_003673.3	HCM, limb-girdle muscular dystrophy
	TMPO	12	Thymopoietin	NM_003276.2	Muscular dystrophy
	TNNC1	3	Troponin C type 1	NM_003280.2	HCM
	TNNI3	19	Troponin I Type 3 (cardiac)	NM_000363.4	HCM, Afib
	TPM1	15	Tropomyosin 1 alpha	NM_000366.5	
	TRPM7	15	Transient receptor potential cation channel, subfamily M, member 7	NM_001301212.1	Amyotrophic lateral sclerosis-parkinsonism/dementia complex
	TSFM	12	Ts translation elongation factor, mitochondrial	NM_001172695.1	Combined oxidative phosphorylation deficiency, fatal mitochondrial disease

Supp. Data Table S2 Investigated genes and associated disease (part 2).

Disease	Gene	CHR	Protein name	RefSeq-Nr.	Other associated diseases
Cardiomyopathies					
	TTR	18	Transthyretin	NM_000371.3	Hyperthyroxinemia, dystransthyretinemic
	TXNRD2	22	Thioredoxin reductase 2	NM_006440.4	
	XK	X	x-linked Kx blood group (McLeod syndrome)	NM_021083.2	
	ZASP	7	ZO-2 associated speckle protein	NM_001289933.1	HCM, myofibrillar myopathy
HCM	*ACTC1*	15	Actin, alpha, cardiac muscle 1	NM_005159.4	ASD, DCM, LVNC
	ANKRD1	10	Ankyrin repeat domain 1 (cardiac muscle)	NM_014391.2	DCM
	CALR3	19	Calreticulin 3	NM_145046.4	
	CAV3	3	Caveolin 3	NM_001234.4	LQTS, limb-girdle muscular dystrophy
	CSRP3	11	Cysteine- and glycine-rich protein 3 (cardiac LIM protein)	NM_003476.4	DCM
	JPH2	20	Junctophilin 2	NM_020433.4	Afib
	LAMP2	X	Lysosome associate membrane glycoprotein 2	NM_001122606.1	GSD
	MAP2K1	15	Mitogen-activated protein kinase 1	NM_002755.3	
	MAP2K2	19	mitogen-activated protein kinase 2	NM_030662.3	Neurofibromatosis-Noonan syndrome
	MRPL3	3	mitochondrial ribosomal protein L3	NM_007208.3	Combined oxidative phosphorylation deficiency
	MYBPC3	11	Myosin binding protein C, cardiac	NM_000256.3	DCM, LVC
	MYH6	14	Myosin, heavy chain, cardiac a	NM_002471.3	ASD, DCM
	MYH7	14	Myosin Heavy chain 7, cardiac B	NM_000257.3	DCM, myosin storage myopathy
	MYL2	12	Myosin light chain 2, regulatory, cardiac	NM_000432.3	DCM
	MYH11	16	Myosin heavy chain 11, smooth muscle	NM_001040114.1	TAAD,
	MYL3	3	Myosin light chain 3, ventricular, skeletal, slow	NM_000258.2	DCM
	MYLK	3	Myosin light chain kinase	NM_053025.3	TAAD,
	MYLK2	20	Myosin light chain kinase 2	NM_033118.3	
	MYOZ2	4	Myozenin 2	NM_016599.4	DCM
	MYOM1	18	Myomesin 1	NM_003803.3	
	PRKAG2	7	Protein kinase, AMP-activated, 2 noncatalytic subunit	NM_016203.3	Glycogen storage disease of heart
	PDLIM3	4	PDZ and LIM domain 3	NM_014476.5_	DCM, HCM
	SLC25A3	12	Solute carrier family 25 (mitochondrial carrier, phosphate carrier) member 3	NM_145305.2	
	TNNT2	1	Troponin T type 2 (cardiac)	NM_001276345.1	DCM, LVNC
	TPM1	15	Tropomiosin 1(alpha)	NM_000366.5	DCM, LVNC
	TTN	2	Titin	NM_001267550.2	DCM, limb-girdle muscular dystrophy
	VCL	10	Vincluin	NM_014000.2	DCM
LVNC	*DTNA*	18	Dystrobrevin, alpha	NM_001390.4	DCM
Cardiac ion channelopathies					
Afib	*CAMK2G*	10	Calcium/calmodulin-dependent protein kinase II gamma	NM_172171.2	
	GATA5	20	GATA-binding protein 5	NM_080473.4	DCM
	GATA6	18	GATA binding protein 6	NM_005257.5	ASD, tetralogy of Fallot
	GJA5	1	Gap junction protein alpha 5	NM_005266.6	
	GJD4	10	Gap junction protein delta 4	NM_153368.2	
	KCNA5	12	Potassium voltage-gated channel, shaker-related subfamily, member 5	NM_002234.3	ASD, LDS
	RANGRF	17	RAN guanine nucleotide release factor	NM_016492.4	BrS
	SCN2B	11	Na channel Auxiliary B subunit type 2	NM_004588.4	ASD, BrS
BrS	*CACNA1C*	12	Calcium channel, voltage-dependent, L type, a 1C subunit	NM_199460.3	
	CACNA2D1	7	Calcium channel, voltage-dependent, a 2/d subunit 1	NM_000722.2	LDS, SQTS
	CACNB2	10	Calcium channel, voltage-dependent, b 2 subunit	NM_000724.3	
	GPD1L	3	Glycerol-3-phosphate dehydrogenase 1–like	NM_015141.3	SIDS
	HCN4	15	Hyperpolarization-activated cyclic nucleotide–gated potassium channel 4	NM_005477.2	SIDS, Afib
	HCN2	19	Hyperpolarization activated cyclic nucleotide-gated potassium channel 2	NM_001194.3	

Supp. Data Table S2 Investigated genes and associated disease (part 3).

Disease	Gene	CHR	Protein name	RefSeq-Nr.	Other associated diseases
Cardiac ion channelopathies					
	KCND3	1	Potassium voltage-gated channel, Shal-related family, member 3	NM_004980.4	SIDS, Afib
	KCNE3	11	Potassium voltage-gated channel, Isk-related family, member 3	NM_005472.4	Paramyotonia congenita
	KCNJ8	12	Potassium inwardly rectifying channel, subfamily J, member 8	NM_004982.3	ASD, LDS, SIDS
	SCN1B	19	Sodium voltage-gated channel, beta subunit 1	NM_199037.3	Afib
	SCN3B	11	Sodium voltage-gated channel, beta subunit 2	NM_018400.3	Afib, SIDS
	SCN4B	11	Sodium voltage-gated channel, beta subunit 4	NM_174934.3	Afib, SIDS
	SCN5A	3	Sodium voltage-gated channel, type 5	NM_001099404.1	ARVC, Afib, DCM, LQTS, SIDS
	SLMAP	3	Sarcolemma associated protein	NM_001304420.2	
	SCN10A	3	Sodium channel type 10	NM_001293306.2	Afib,
	TRPM4	19	Transient receptor potential cation channel, subfamily M, member 4	NM_017636.3	Familial progressive cardiac conduction defect
CPVT	*CASQ2*	1	Calsequestrin 2 (cardiac muscle)	NM_001232.3	HCM
	RYR2	1	Ryanodine receptor	NM_001035.2	ARVC, LQTS
	TRDN	6	Triadin	NM_006073.3	DCM, LQTS
IVF	*ABCC8*	11	ATP-binding cassette, subfamily C (CFTR/MRP), member 8	NM_001287174.1	Familial hyperinsulinemic hypoglycemia, hypoglycemia of infancy
	DPP6	7	Dipeptidyl-peptidase 6	NM_130797.3	
	MED23	6	Mediator complex subunit 23	NM_004830.3	Intellectual disability
	SEMA3A	7	Sema domain, immunoglobulin domain (Ig), short basic domain secreted 3A	NM_006080.2	BrS, Kallmann syndrome
LQTS	*AKAP9*	7	A kinase (PRKA) anchor protein (yotiao) 9	NM_001148.4	BrS, LDS
	ANK2	4	Ankyrin 2	NM_001148.4	
	CALM1	14	Calmodulin 1	NM_006888.4	SIDS, CPVT
	CALM2	2	Calmodulin 2	NM_001743.4	SIDS, CPVT
	CALM3	19	Calmodulin 3	NM_005184.2	SIDS, CPVT
	DLG1	3	Discs, large homolog 1	NM_001098424.1	
	KCNE1	21	Potassium voltage-gated channel, Isk-related family, member 1	NM_000219.5	Afib
	KCNE1L	X	KCNE1-like	NM_012282.2	Afib
	KCNE2	21	Potassium voltage-gated channel, Isk-related family, member 2	NM_172201.1	
	KCNE5	X	Potassium voltage-gated channel, subfamily E	NM_012282.2	
	KCNH2	7	Potassium Voltage gated channel, subfamily H, member 2	NM_000238.3	
	KCNJ2	17	Potassium inwardly rectifying channel, subfamily J, member 2	NM_000891.2	Afib, SQTS
	KCNJ5	11	Potassium inwardly rectifying channel, subfamily J, member 5	NM_000890.3	
	KCNQ1	11	Potassium voltage gated channel, KQT type	NM_000218.2	Afib, SQTS
	NOS1AP	1	Nitric oxide synthase 1 (neuronal) adaptor protein	NM_014697.2	
	SNTA1	20	Syntrophin alpha 1	NM_003098.2	BrS, LDS, SIDS
Cardiovascular diseases					
AOVD	*NOTCH1*	9	Notch 1	NM_017617.3	
ASD	*GATA4*	8	GATA-binding protein 4	NM_002052.3	
	GATA6	18	GATA-binding protein 6	NM_005257.5	
	GJA1	6	Gap junction protein alpha 1	NM_000165.4	SIDS
	Nkx2-5	5	NK2 transcription factor-related 5	NM_004387.3	Tetralogy of Fallot
	TBX5	12	T-box 5	NM_000192.3	Holt-Oram syndrome
CVDX	*FLNA*	X	Filamin A, alpha	NM_001110556.1	
MVP	*DCHS1*	11	Dachsous cadherin-related 1	NM_003737.3	
Tricuspid atresia	*HEY2*	6	Hes-related family BHLH transcription factor with YRPW motif 2	NM_012259.2	
TAAD	*ACTA2*	10	Actin, alpha 2, smooth muscle, aorta	NM_001141945.1	Multisystemic smooth muscle dysfunction syndrome
	PRKG1	10	Protein kinase, cGMP-dependent type 1	NM_006258.3	Phosphoglycerate kinase deficiency

Supp. Data Table S2 Investigated genes and associated disease (part 4).

Disease	Gene	CHR	Protein name	RefSeq-Nr.	Other associated diseases
Connective tissue diseases					
WMS	*ADAMTS10*	19	ADAM metallopeptidase with thrombospondin type 1 motif 10	NM_030957.3	
EDS	*COL3A1*	2	Collagen, type III, alpha 1	NM_000090.3	
	COL5A1	9	Collagen, type V, alpha 1	NM_000093.4	
	COL5A2	2	Collagen, type V, alpha 2	NM_000393.3	
Cutis laxa	*EFEMP2*	11	EGF containing fibulin-like extracellular matrix protein 2	NM_016938.4	AA, arteriopathy syndrome
	ELN	7	Elastin	NM_001278939.1	
	CTGF	6	Connective tissue growth factor	NM_001901.2	HCM
MS	*FBN1*	15	Fibrillin 1	NM_000138.4	
	FBN2	5	Fibrillin 2	NM_001999.3	
LDS	*TGFβ2*	1	Transforming growth factor beta 2	NM_001135599.2	
	TGFβR1	9	Transforming growth factor, beta receptor 1	NM_004612.2	
	TGFβR2	3	Transforming growth factor, beta receptor 2	NM_001024847.2	
	SMAD3	15	SMAD family member 3	NM_005902.3	
Metabolic disease					
Fabry disease	*GLA*	X	Galactosidase, alpha	NM_000169.2	HCM
GA	*ETFA*	15	Electron-transfer-flavoprotein, alpha polypeptide	NM_001127716.1	
	ETFB	19	Electron-transfer-flavoprotein, beta polypeptide	NM_001985.2	
	ETFDH	4	Electron-transferring-flavoprotein dehydrogenase	NM_001281738.1	
GKD	*GK*	X	Glycerol kinase	NM_001128127.2	
	GLB1	3	Galactosidase, beta 1	NM_000404.2	
GSD	*AGL*	1	Amylo-alpha-1,6-glucosidase, 4-alpha-glucanontransferase	NM_000028.2	
	G6PC	17	Glucose-6-phosphatase, catalytic subunit	NM_000151.3	
	GUSB	7	Glucuronidase, beta	NM_000181.3	
	SLC37A4	11	Solute carrier family 37 (glucose-6-phosphate transporter)	NM_001164278.1	
HHF	*HADH*	4	Hydroxyacyl-CoA dehydrogenase	NM_001184705.2	
	HADHA	2	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit	NM_000182.4	
	HADHB	2	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, beta subunit	NM_001281512.1	
HMGCS-deficiency	*HMGCL*	1	3-hydroxymethyl-3-methylglutaryl-CoA lyase	NM_001166059.1	
	HMGCS2	1	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	NM_001166107.1	
MCAD	*ACADM*	1	Acyl-CoA dehydrogenase, C-4 to C-12 straight chain	NM_001127328.2	
	ACAD9	3	Acyl-CoA dehydrogenase family, member 9	NM_014049.4	Muscular dystrophy
	ACADS	12	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	NM_001302554.1	
	ACADVL	17	Acyl-CoA dehydrogenase, very long chain	NM_001270447.1	
mt-D	*ATP5E*	20	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	NM_006886.3	
	COA5	2	Cytochrome c oxidase assembly factor 5	NM_001008215.2	Fatal infantile cardioencephalomyopathy
	FOXRED1	11	FAD-dependent oxidoreductase domain containing 1	NM_017547.3	
	SCO2	22	SCO2 cytochrome c oxidase assembly protein	NM_001169109.1	
	SLC25A3	12	Solute carrier family 25 (mitochondrial carrier, phosphate carrier) member 3	NM_145305.2	Carnitine-acylcarnitine translocase deficiency
SPCD	*CPT1A*	11	Carnitine palmitoyltransferase 1A (liver)	NM_001031847.2	
	CPT2	1	Carnitine palmitoyltransferase 2	NM_000098.2	
	SLC22A5	5	Solute carrier family 22 (organic cation/carnitine transporter), member 5	NM_001270888.1	

Supp. Data Table S2 Investigated genes and associated disease (part 5).

Disease	Gene	CHR	Protein name	RefSeq-Nr.	Other associated diseases
Respiratory diseases					
CCHS	*ASCL1*	12	Achaete-scute family bHLH transcription factor 1	NM_004316.3	
PAH	*ACVRL1*	12	Activin A receptor type II-like 1	NM_000020.2	
	BMPR1B	4	Bone morphogenetic protein receptor, type IB	NM_001256793.1	
	BMPR2	2	Bone morphogenetic protein receptor, type II	NM_001204.6	
	CAV1	7	Caveolin 1	NM_001753.4	
	ENG	9	Endoglin	NM_001114753.2	
	SMAD9	13	SMAd family member 9	NM_001127217.2	
Muscular dystrophy					
SCD	*ZNF365*	10	Zinc finger protein 365	NM_199451.2	
CFC1	*BRAF*	7	V-raf murine sarcoma viral oncogene homolog B	NM_004333.4	HCM
NSLL	*CBL*	11	Cbl proto-oncogene, E3 ubiquitin protein ligase	NM_005188.3	HCM
DM1	*DMPK*	19	Dystrophia myotonica-protein kinase	NM_001288764.1	DCM
CMD	*FKRP*	19	Fukutin related protein	NM_001039885.2	
EDDM	*SYNE1*	6	Spectrin repeat containing, nuclear envelope 1	NM_033071.3	
	EMD	X	Emerin (Emery-Dreifuss muscular dystrophy)	NM_000117.2	
	SYNE2	14	Spectrin repeat containing, nuclear envelope 2	NM_015180.4	
	HFE	6	Hemochromatosis	NM_000410.3	
	ECE1	1	Endothelin converting enzyme 1	NM_001113347.1	Hirschsprung disease, cardiac defects, and autonomic dysfunction
EMPF	*DNM1L*	12	Dynamin 1-like	NM_001278464.1	

Abbreviations:

AA: aortic aneurysm, Afib: atrial fibrillation, AOVD: aortic valve disease, ARVC: arrhythmogenic right ventricular cardiomyopathy, ASD: atrial septal defect, ATS: arterial tortuosity syndrome, BrS: Brugada syndrome, BTHS: Barth syndrome, CCHS: congenital central hypoventilation syndrome, CFC1: cardiofaciocutaneous syndrome 1, CMD: congenital muscular dystrophy, CPVT: catecholaminergic polymorphic ventricular tachycardia, CVDX: cardiac valvular dysplasia x-linked, DCM: dilated cardiomyopathy, DM1: myotonic dystrophy type 1, EDS: Ehlers-Danlos syndrome, EDMD: Emery-Dreifuss muscular dystrophy, EMPF: encephalopathy due to defective mitochondrial and peroxisomal fission, GA: glutaric aciduria, GKD: glycerol kinase deficiency, GSD: glycogen storage disease, HHF: familial hyperinsulinemic hypoglycemia, HCM: hypertrophic cardiomyopathy, HMGCS: HMG-CoA synthase deficiency, IVF: idiopathic ventricular fibrillation, LDS: Loeys-Dietz syndrome, LQTS: long QT syndrome, LVNC: left ventricular noncompaction cardiomyopathy, MCAD: medium-chain-acyl-CoA-dehydrogenase deficiency, MS: Marfan syndrome, mt-D: mitochondrial defects, MVP: mitral valve prolapse, NSLL: Noonan syndrome-like disorder with or without juvenile myelomonocytic leukemia, PAH: pulmonary hypertension, SCD: sudden cardiac death, SIDS: sudden infant death syndrome, SPCD: systemic primary carnitine deficiency, SQTS: short QT syndrome, SSS: sick sinus syndrome 2, TAAD: familial thoracic aortic aneurysm and aortic dissection, WMS: Weill Marchesani syndrome.

Supp. Data Table S3 Average coverage per gene (part 1).

Genes	Average	STDEV	Median	Min.	Max.
ABCC8	197.59	59.55	195.44	52.87	531.02
ABCC9	81.32	26.90	77.17	19.51	198.09
ACAD9	108.90	34.01	106.37	29.17	294.63
ACADM	74.91	23.07	71.96	18.53	199.60
ACADS	166.63	62.00	155.01	40.73	451.78
ACADVL	72.41	23.59	69.03	18.09	161.00
ACTA2	121.21	35.09	118.73	34.28	291.74
ACTC1	69.35	21.04	66.28	17.11	171.16
ACTN2	150.93	43.54	148.83	39.74	410.84
ACVRL1	40.64	12.57	39.02	10.10	102.43
ADAMTS10	131.64	50.45	124.98	34.58	390.09
AGL	17.48	5.82	16.58	4.02	42.50
AKAP9	55.07	16.92	52.67	13.72	141.91
ANK2	89.41	27.29	88.11	24.23	226.15
ANKRD1	97.88	31.08	92.58	22.25	244.75
ASCL1	67.68	28.01	63.56	15.18	200.40
ATP5E	110.94	36.09	106.30	26.90	264.77
BAG3	127.28	39.44	120.95	30.98	316.81
BMPR1B	188.14	72.30	186.94	50.22	488.84
BMPR2	114.21	35.58	112.13	29.06	329.38
BRAF	207.84	76.92	211.29	60.90	601.99
CACNA1C	11.25	3.84	11.21	3.40	30.63
CACNA2D1	294.91	115.78	291.12	83.76	739.21
CACNB2	40.65	14.71	41.56	12.33	115.95
CALM1	88.78	28.87	87.49	22.37	227.44
CALM2	113.27	33.55	108.83	28.92	290.39
CALM3	157.37	54.21	153.87	38.44	418.17
CALR3	229.72	89.29	226.00	53.39	738.34
CAMK2G	27.85	8.47	27.26	8.03	75.36
CASQ2	140.54	43.92	139.52	39.50	366.85
CAV1	26.67	7.58	26.02	6.41	65.93
CAV3	76.23	22.71	75.09	19.52	205.87
CBL	81.76	23.85	81.31	21.86	221.85
COA5	73.04	22.31	69.67	16.06	175.22
COL3A1	88.72	35.66	80.89	20.70	259.06
COL5A1	292.39	96.98	279.25	80.73	796.17
COL5A2	118.65	41.32	112.07	29.00	303.64
CPT1A	123.71	42.42	122.97	31.34	372.19
CPT2	95.54	29.05	92.68	24.46	250.24
CRYAB	180.73	53.55	178.43	51.28	400.06
CSRP3	77.78	22.79	75.53	20.99	203.42
CTF1	34.02	11.26	32.96	9.19	91.29
CTGF	65.70	20.29	64.23	15.64	149.64
CTNNA3	530.58	246.37	537.91	158.58	1422.98
DCHS1	102.84	31.80	100.90	26.36	250.36
DES	124.21	44.82	117.50	32.31	347.53
DLG1	44.60	13.98	43.54	11.64	117.86
DMD	23.96	12.10	22.23	6.21	64.35

Genes	Average	STDEV	Median	Min.	Max.
DMPK	32.21	11.54	29.97	8.92	82.13
DNAJC19	16.81	5.27	16.60	4.01	43.30
DNM1L	53.88	16.80	52.40	13.68	149.26
DOLK	89.86	33.62	84.39	22.22	217.63
DPP6	248.08	102.30	254.46	77.29	717.59
DSC2	42.35	14.25	40.12	10.26	105.49
DSG2	74.52	22.73	72.74	19.80	190.92
DSP	58.44	17.75	56.22	14.96	143.98
DTNA	16.71	5.77	16.54	4.89	42.40
ECE1	54.26	18.14	53.74	15.54	152.59
EFEMP2	74.48	25.25	70.33	20.37	168.42
ELN	54.47	20.55	51.62	12.84	161.25
EMD	94.82	55.48	77.02	22.78	380.76
ENG	77.00	26.20	74.26	21.52	209.39
ETFA	244.72	75.40	241.12	68.90	705.47
ETFB	89.71	31.21	89.77	25.42	272.35
ETFDH	125.40	37.15	120.75	31.42	307.38
EYA4	52.89	18.57	52.83	15.62	132.94
FBN1	160.44	48.29	155.48	41.48	393.66
FBN2	162.05	48.24	157.23	41.74	385.52
FHL2	76.49	22.84	76.30	20.73	206.01
FKRP	27.21	10.65	25.26	6.91	70.96
FKTN	34.66	10.80	33.49	8.78	85.05
FLNA	57.37	31.84	50.63	17.58	227.06
FOXRED1	45.61	15.06	44.79	11.13	116.16
G6PC	68.80	21.23	68.48	17.15	188.89
GAA	43.29	15.19	41.09	11.26	108.82
GATA4	136.72	41.83	132.54	40.06	380.13
GATA5	104.94	39.56	97.66	29.15	262.39
GATA6	85.97	27.08	82.64	22.25	225.14
GATA6	85.97	27.08	82.64	22.25	225.14
GATAD1	41.89	12.96	39.97	10.55	104.39
GJA1	95.85	29.35	91.63	23.32	246.78
GJA5	53.80	16.84	52.43	13.54	132.13
GJD4	75.01	27.35	70.59	18.06	197.61
GK	27.16	12.42	23.59	6.83	73.98
GLA	73.86	36.06	62.47	21.04	225.34
GLB1	123.00	35.74	120.34	32.48	334.35
GPD1L	124.10	35.51	121.21	32.47	324.76
GUSB	139.33	49.34	137.68	38.86	420.16
HADH	87.75	24.92	85.54	22.71	217.63
HADHA	152.68	45.07	150.74	40.51	416.89
HADHB	161.77	50.36	157.31	41.51	452.50
HCN2	83.59	36.57	79.48	25.05	246.81
HCN4	92.56	31.01	87.80	24.73	251.25
HEY2	102.51	30.92	98.48	25.73	237.11
HFE	12.73	3.90	12.33	3.29	31.69
HMGCL	105.57	33.79	101.97	25.58	297.08

Supp. Data Table S3 Average coverage per gene (part 2).

Genes	Average	STDEV	Median	Min.	Max.
HMGCS2	44.16	12.44	43.34	11.71	107.12
ILK	49.43	14.95	47.46	12.67	112.42
JPH2	76.34	25.22	75.19	21.24	219.85
JUP	70.75	24.88	69.47	19.08	200.28
KCNA5	98.55	30.51	93.72	32.33	124.81
KCND3	161.83	66.13	165.39	51.09	453.10
KCNE1	32.35	9.61	32.07	9.25	85.69
KCNE1L	48.38	26.69	40.44	13.12	188.08
KCNE2	87.84	25.77	87.34	24.63	239.87
KCNE3	53.93	15.87	52.95	13.86	142.18
KCNE5	37.29	9.82	36.52	12.54	47.64
KCNH2	38.98	13.54	37.52	10.28	104.65
KCNJ2	23.31	7.11	22.52	5.84	59.50
KCNJ5	185.94	55.32	183.13	48.76	463.39
KCNJ8	74.60	23.39	71.35	20.62	172.86
KCNQ1	208.08	83.41	206.53	68.02	551.71
LAMA4	46.46	13.88	44.56	12.05	110.17
LAMP2	22.57	10.15	19.88	6.13	61.19
LDB3	38.31	11.63	37.35	10.08	104.76
LMNA	51.23	16.76	48.59	13.94	136.75
MAP2K1	238.98	79.18	239.97	70.42	686.97
MAP2K2	234.64	88.92	227.11	67.76	677.44
MED23	88.58	29.73	82.96	20.47	218.79
MRPL3	136.16	43.41	133.10	34.32	333.80
MYBPC3	173.27	60.89	165.63	44.12	467.02
MYH11	64.17	22.09	63.54	17.42	195.73
MYH6	138.33	43.01	136.93	37.60	371.27
MYH7	133.77	41.51	131.21	35.01	350.08
MYL2	214.89	74.24	210.39	50.71	653.85
MYL3	136.39	45.36	128.74	38.12	365.97
MYLK	55.96	15.99	54.66	16.51	143.79
MYLK2	129.14	41.72	123.80	33.63	342.21
MYOM1	90.30	26.89	89.44	24.51	246.97
MYOZ2	102.02	33.27	97.60	27.75	239.91
MYPN	147.40	44.01	145.44	38.43	407.71
NEBL	75.63	23.82	75.66	22.00	195.51
NEXN	52.98	17.09	52.31	14.16	151.74
NKX2-5	75.36	28.87	71.45	36.35	108.58
NOS1AP	82.87	28.07	82.80	25.14	212.50
NOTCH1	126.23	45.18	115.30	32.16	311.28
PDLIM3	48.58	13.64	48.12	12.30	114.92
PKP2	64.89	21.21	64.65	17.58	188.63
PLN	81.87	29.15	76.78	19.14	197.34
PRKAG2	178.98	67.59	178.20	57.11	504.62
PRKG1	350.62	153.71	349.45	106.30	905.19
PSEN1	56.51	17.03	55.70	15.14	156.47
PSEN2	81.66	24.36	79.61	22.85	212.22
RAF1	190.88	58.84	186.79	50.66	551.82

Genes	Average	STDEV	Median	Min.	Max.
RANGRF	28.20	9.98	26.92	7.15	62.92
RBM20	152.84	44.91	153.15	45.13	396.50
RYR2	207.27	65.61	206.53	58.03	558.86
SCN10A	149.58	43.04	147.11	40.61	384.91
SCN1B	66.26	22.09	62.54	17.90	170.92
SCN2B	96.39	33.56	93.29	24.56	258.03
SCN3B	31.08	9.58	30.70	7.64	85.25
SCN4B	19.19	5.98	18.70	4.77	48.76
SCN5A	22.52	6.72	22.15	6.26	60.25
SCO2	37.26	14.84	35.28	9.58	87.64
SDHA	168.60	51.44	163.48	45.76	491.09
SEMA3A	128.05	43.74	122.63	33.58	329.63
SGCD	58.85	22.69	58.61	17.63	156.79
SLC22A5	104.17	28.56	100.75	28.06	253.97
SLC25A3	54.15	17.27	52.80	13.74	135.20
SLC25A3	54.15	17.27	52.80	13.74	135.20
SLC37A4	18.99	5.87	18.43	4.67	47.22
SLMAP	243.95	73.76	240.10	63.82	652.42
SMAD3	39.19	11.48	39.63	11.53	102.04
SMAD9	43.26	12.89	42.54	12.32	115.78
SNTA1	131.27	48.78	129.58	37.60	422.86
SYNE1	78.74	23.21	76.11	19.96	195.10
SYNE2	69.35	20.28	68.19	18.40	183.58
TAZ	21.09	11.25	17.92	6.37	77.94
TBX5	39.96	12.24	39.03	10.59	110.20
TCAP	56.19	20.62	52.48	13.29	144.18
TGFB2	48.70	14.63	47.98	13.20	123.44
TGFB3	103.54	31.84	103.42	26.76	280.50
TGFBR1	38.50	12.39	36.12	8.42	92.26
TGFBR2	54.99	15.91	53.33	15.47	132.20
TMEM43	124.29	37.14	121.05	33.43	304.92
TMPO	44.12	13.94	42.68	10.34	115.88
TNNC1	206.93	67.54	197.05	49.45	524.48
TNNI3	178.17	68.81	166.76	45.59	490.88
TNNT2	94.95	29.48	92.29	25.71	241.38
TPM1	53.53	15.40	52.63	13.93	131.99
TPM1	53.53	15.40	52.63	13.93	131.99
TRDN	234.93	90.38	231.79	64.85	633.54
TRPM4	104.08	38.53	101.29	26.75	314.37
TRPM7	89.35	28.25	86.85	23.09	250.58
TSFM	66.72	21.11	64.09	17.08	176.69
TTN	32.74	11.38	30.80	7.81	77.87
TTR	162.91	52.27	155.53	36.56	411.55
TXNRD2	414.15	137.27	405.08	111.05	1149.59
VCL	72.83	21.28	72.26	20.45	197.28
XK	41.57	18.76	35.12	9.93	106.02
ZASP	38.62	8.69	35.87	28.63	52.96
ZNF365	89.30	30.62	89.05	26.56	233.83

Calculation: average coverage of the gene = (number of total reads mapped to exons* read length – overlapping bases) / (sum of exon lengths).

Abbreviations: STDEV: standard deviation.

Supp. Data Table S4 VUS4-VUS0 in our SIDS cohort (part 1).

Supp. Data Table S4 VUS4-VUS0 in our SIDs cohort.

Cas	Gender	Age [mo]	SIDS category	rs-Nr.	Gene	HGVS RefSeq-Nr.	genomic	HGVS RefSeq-Nr.	Coding Effect	Location	cDNA	Protein change	dbSNP ALLMAF	espEAMAF	Human [HGMD]-Phenotype	Mutation	Database	HGMD Variant class	associated disease	AGVGD	SIFT	MAPP	MutationTaster	Grantham distance	polyphen2	ExAC EURMAF (non-finnish)	1000 Genomes EURMAF	Coverage	Alternative Allele	Sanger confirmed	in-house database	subclass based on scoring scheme
SIDS001	F	9	2	rs17848485	*CPT2*	NG_008035.1	NM_0000958.2		missense	exon	c.1634A>C	p.(Glu545Ala)	0.0010	0.0000	Hypercholesterolaemia	DM?	SPCD	C65	Deleterious	bad	disease-causing	107	probably damaging	0.0000	0.0030	34	0.38	√	NA	V053
SIDS001	F	9	2	rs144848998	*4MK2*	NG_0090006.2	NM_0011484.4		missense	exon	c.623 8G>T	p.(Lys2076Asn)	0.0000	0.0000	-	-	LQTS	C0	Deleterious	good	disease-causing	94	possibly damaging	0.0000	0.0010	62	0.37	NA	NA	V052
SIDS001	F	9	2	rs149331873	*4GL*	NG_0128655.1	NM_0000282.2		missense	exon	c.2657>T	p.(Pro886Leu)	0.0000	0.0000	-	-	GSD	C0	Tolerated	unknown	disease-causing	98	benign	0.0000	0.0000	87	0.43	NA	NA	V051
SIDS001	F	9	2	rs200130356	*FLNA*	NG_011506.1	NM_0011105561		missense	exon	c.4451A>G	p.(Gln1484Arg)	0.0010	0.0015	Mental retardation	DM	heterotopia	C35	Deleterious	bad	polymorphism	43	benign	0.0016	0.0000	25	0.4	NA	NA	V051
SIDS004	M	7	2	rs199583774	*PKP2*	NG_0090001	NM_0045723		missense	exon	c.2083C>T	p.(Arg695Cys)	0.0000	0.0000	-	-	ARVC	C25	Deleterious	bad	disease-causing	180	probably damaging	0.0001	0.0010	70	0.43	NA	NA	V054
SIDS005	M	4	1	rs142890619	*CQL5A1*	NG_0080301	NM_0000933		missense	exon	c.598G>A	p.(Asp200Asn)	0.0000	0.0010	-	-	EDS	C0	Deleterious	bad	disease-causing	23	probably damaging	0.0000	0.0010	107	0.47	NA	NA	V053
SIDS007	F	8	2	rs143641133	*DOLK*	NG_0170091	NM_0149083		missense	exon	c.1324G>A	p.(Ala442Thr)	0.0000	0.0000	-	-	DCM	C0	Deleterious	bad	disease-causing	58	probably damaging	0.0000	0.0010	131	0.36	√	NA	V053
SIDS007	F	8	2	rs121913013	*DSG2*	NG_0070723	NM_0019433		missense	exon	c.1666G>A	p.(Val56Met)	0.0000	0.0035	ARVC	DM	ARVC	C0	Deleterious	good	disease-causing	105	probably damaging	0.0006	0.0010	46	0.35	NA	NA	V051
SIDS008	F	4	1	rs145425478	*DTNA*	NG_0092011	NM_0013904		missense	exon	c.1757C>T	p.(Pro586Leu)	0.0000	0.0000	-	-	LVNC	C0	Deleterious	good	disease-causing	98	probably damaging	0.0000	NA	54	0.5	NA	NA	V051
SIDS008	F	4	1	rs200130356	*FLNA*	NG_011506.1	NM_0011105561		missense	exon	c.4451A>G	p.(Gln1484Arg)	0.0010	0.0015	Mental retardation	DM	heterotopia	C35	Deleterious	bad	polymorphism	43	benign	0.0016	0.0000	43	0.47	NA	NA	V051
SIDS009	M	2	2	rs151254520	*4CADVL*	NG_0079751	NM_0012704471		missense	exon	c.1363C>T	p.(Arg655Cys)	0.0000	0.0002	-	-	MCAD	C0	Deleterious	bad	disease-causing	180	probably damaging	0.0006	NA	123	0.32	NA	NA	V052
SIDS009	M	2	2	~	*SMAD9*	NG_0169631	NM_0011272172		nonsense	exon	c.811C>T	p.(Gln271*)	NA	NA	-	-	PAH	NA	NA	NA	disease-causing	NA	NA	NA	NA	58	0.41	NA	0.0000	V052
SIDS010	F	3	2	rs200473206	*DSP*	NG_0088031	NM_0044152		splice site	splice site	c.273+5G>A	p.(?)	0.0000	0.0005	ARVC	DM	ARVC	NA	NA	NA	NA	NA	NA	0.0005	NA	62	0.48	NA	NA	V053
SIDS010	F	3	2	rs141121553	*DCHS1*	NG_0338581	NM_0037372		missense	exon	c.6062G>A	p.(Arg2021His)	0.0000	0.0000	-	-	MVP	C25	Deleterious	bad	polymorphism	29	benign	0.0000	0.0000	94	0.41	NA	NA	V052
SIDS010	F	3	2	rs117360770	*SYNE1*	NG_0128551	NM_1829612		missense	exon	c.9148C>G	p.(Leu3050Val)	0.0020	0.0010	-	-	EDMD	C25	Deleterious	bad	NA	32	probably damaging	0.0020	0.0020	115	0.46	NA	NA	V051
SIDS011	M	2	1	rs72352737	*TRPM4*	NG_0275511	NM_0176363		nonsense	exon	c.1575G>A	p.(Trp525*)	0.0010	0.0011	-	-	Brs	NA	NA	NA	disease-causing	NA	NA	0.0018	0.0010	23	0.48	√	NA	V054
SIDS012	M	10	2	rs145850743	*CQL5A2*	NG_0117991	NM_0009333		missense	exon	c.1301C>T	p.(Thr434Met)	0.0000	0.0026	-	-	EDS	C65	Deleterious	bad	disease-causing	81	benign	0.0020	0.0000	30	0.5	NA	NA	V052
SIDS013	M	3	1	rs200015085	*SYNE2*	NG_0289221	NM_0073733		missense	exon	c.610A>G	p.(Ile204Val)	0.0000	0.0000	-	-	Noonan syndrome	C25	Deleterious	bad	disease-causing	29	benign	0.0000	0.0000	111	0.48	NA	NA	V052
SIDS014	M	3	1	rs141821444	*SYNE1*	NG_0128551	NM_1829612		missense	exon	c.9448G>A	p.(Ala3150Thr)	0.0000	0.0000	-	-	EDMD	C55	Deleterious	bad	NA	58	probably damaging	0.0000	NA	51	0.43	NA	NA	V051
SIDS015	M	1	2	rs143788120	*MRP13*	NG_0292071	NM_0072083		missense	exon	c.862T>C	p.(Ser288Pro)	0.0000	0.0000	-	-	HCM	C65	Deleterious	bad	disease-causing	74	probably damaging	0.0006	0.0030	115	0.43	NA	NA	V053
SIDS016	M	8	1	rs142992009	*MYH6*	NG_0234441	NM_0024713		missense	exon	c.1763A>C	p.(Asp588Ala)	0.0000	0.0022	-	-	HCM	C65	Deleterious	bad	disease-causing	126	benign	0.0030	0.0030	108	0.4	NA	NA	V052
SIDS016	M	8	1	rs201082652	*SLC22A5*	NG_0089822	NM_0030603		missense	exon	c.364G>T	p.(Asp122Tyr)	0.0000	0.0003	systemic primary carnitine deficiency	DM	SPCD	C0	Deleterious	good	NA	160	probably damaging	0.0005	0.0000	74	0.45	NA	NA	V052
SIDS021	M	4	1	~	*TSPAN*	NG_0169711	NM_0011726961		Frameshift	exon	c.971_972del	p.(Gln324Argfs*11)	NA	0.0000	-	-	DCM	NA	NA	NA	NA	NA	NA	NA	NA	40	0.4	√	0.0000	V053
SIDS021	M	4	1	rs147589613	*CQL5A1*	NG_0080301	NM_0000934		missense	exon	c.341C>A	p.(Ala114Asp)	0.0000	0.0010	-	-	EDS	C0	Deleterious	bad	NA	126	disease-causing	0.0000	0.0000	73	0.47	NA	NA	V052
SIDS021	M	4	1	rs183156491	*NOTCH1*	NG_0074581	NM_0176173		missense	exon	c.4028C>T	p.(Ala1343Val)	0.0010	0.0010	Aortic valve disease	DM	AOVD	C0	Deleterious	bad	disease-causing	64	possibly damaging	0.0020	0.0010	36	0.5	NA	NA	V051
SIDS023	M	4	2	rs199473233	*SCN5A*	NG_0089341	NM_0010994041		missense	exon	c.4057G>A	p.(Val1353Met)	0.0000	0.0000	Brugada syndrome	DM	Brs, HCM, LQTS	C15	Deleterious	bad	disease-causing	21	probably damaging	0.0000	NA	110	0.46	NA	NA	V051
SIDS024	M	2	2	rs140470576	*4KAP9*	NG_0116231	NM_0057514		missense	exon	c.1011 8C>A	p.(Ser337Tyr)	0.0000	0.0020	-	-	LQTS	C65	Deleterious	bad	disease-causing	144	probably damaging	0.0000	0.0000	84	0.48	NA	NA	V054
SIDS024	M	2	2	rs121913013	*DSG2*	NG_0070723	NM_0019433		missense	exon	c.1666G>A	p.(Val556Met)	0.0000	0.0035	ARVC	DM	ARVC	C0	Deleterious	good	disease-causing	21	probably damaging	0.0006	0.0010	28	0.46	NA	NA	V051
SIDS024	M	2	2	rs150290283	*SYNE1*	NG_0128551	NM_1829612		missense	exon	c.17089C>T	p.(Arg5697Trp)	0.0000	0.0000	-	-	EDMD	C65	Deleterious	bad	NA	101	possibly damaging	0.0000	NA	51	0.47	NA	NA	V051
SIDS025	F	3	2	rs200742016	*SYNE2*	NG_0117561	NM_1829142		missense	exon	c.9230C>T	p.(Pro3077Leu)	0.0010	0.0019	-	-	EDMD	C65	Deleterious	bad	disease-causing	98	probably damaging	0.0017	0.0020	50	0.5	NA	NA	V052
SIDS029	M	7	1	~	*DCHS1*	NG_0338581	NM_0037372		nonsense	exon	c.4672C>T	p.(Gln1558*)	NA	NA	-	-	MVP	NA	NA	NA	disease-causing	NA	NA	NA	NA	148	0.45	NA	0.0000	V052
SIDS029	M	7	1	rs181059922	*SYNE2*	NG_0117561	NM_1829142		missense	exon	c.15865G>A	p.(Val5289Met)	0.0000	0.0000	-	-	EDMD	C0	Deleterious	good	NA	NA	disease-causing	21	possibly damaging	0.0000	0.0000	87	0.38	NA	NA	V052
SIDS029	M	7	1	rs141443652	*TMPO*	NG_0213931	NM_0032762		missense	exon	c.1277C>T	p.(Pro426Leu)	0.0010	0.0015	Muscular dystrophy	DM	DCM	C0	Deleterious	bad	disease-causing	98	probably damaging	0.0020	0.0010	41	0.39	NA	NA	V052
SIDS031	F	4	2	rs141131535	*MYLK*	NG_0291111	NM_0530253		missense	exon	c.3446>A	p.(Arg1151His)	0.0000	0.0002	-	-	HCM	C25	Deleterious	bad	disease-causing	29	possibly damaging	0.0001	0.0010	38	0.37	√	NA	V053
SIDS031	F	4	2	rs373665895	*RYR2*	NG_0087992	NM_0010352		missense	exon	c.2828T>C	p.(Leu943Ser)	0.0000	0.0001	-	-	CPVT	C65	Deleterious	bad	disease-causing	145	benign	0.0003	NA	84	0.49	NA	NA	V052
SIDS031	F	4	2	rs35912339	*MYLK*	NG_0291111	NM_0530253		missense	exon	c.1007C>T	p.(Pro336Leu)	0.0000	0.0013	-	-	HCM	C15	Deleterious	bad	polymorphism	98	possibly damaging	0.0017	0.0020	53	0.49	NA	NA	V051
SIDS031	F	4	2	rs752038761	*RYR2*	NG_0087992	NM_0010352		missense	exon	c.4798C>T	p.(Pro1600Ser)	0.0000	NA	-	-	CPVT	C65	Deleterious	bad	disease-causing	74	probably damaging	0.0000	NA	42	0.5	NA	NA	V051
SIDS032	M	3	2	~	*4MK2*	NG_0090062	NM_0011484		missense	exon	c.2966T>A	p.(Leu989His)	NA	0.0000	-	-	LQTS	C65	Deleterious	bad	disease-causing	99	probably damaging	NA	NA	122	0.42	NA	0.0000	V052
SIDS032	M	3	2	rs199909093	*4KAP9*	NG_0116231	NM_0057514		missense	exon	c.2786T>C	p.(Val262Ala)	0.0000	0.0000	-	-	LQTS	C25	Deleterious	good	polymorphism	64	benign	0.0000	0.0010	85	0.45	NA	NA	V051
SIDS037	F	4	1	rs139965373	*4KAP9*	NG_0116231	NM_0057514		missense	exon	c.3580G>A	p.(Ala1194Thr)	0.0010	0.0000	-	-	LQTS	C55	Deleterious	bad	disease-causing	58	probably damaging	0.0000	0.0000	29	0.41	√	NA	V054
SIDS037	F	4	1	rs749544930	*DLG1*	NG_0250991	NM_0040872		missense	exon	c.2455C>G	p.(Arg819Gly)	0.0000	NA	-	-	LQTS	C45	Deleterious	bad	disease-causing	125	probably damaging	0.0000	0.0000	23	0.26	NA	NA	V052
SIDS037	F	4	1	rs199681901	*DSG2*	NG_0070723	NM_0019433		missense	exon	c.1781T>C	p.(Leu594Pro)	0.0000	0.0000	-	-	ARVC	C0	Tolerated	good	disease-causing	98	possibly damaging	0.0005	0.0020	20	0.4	NA	NA	V051
SIDS037	F	4	1	rs138004884	*SYNE1*	NG_0128551	NM_1829612		missense	exon	c.6934T>C	p.(Phe2312Leu)	0.0000	0.0000	-	-	EDMD	C0	Tolerated	bad	NA	22	probably damaging	0.0012	0.0010	121	0.46	NA	NA	V051
SIDS040	F	8	2	rs201571987	*TXNRD2*	NG_0118351	NM_0064404		missense	exon	c.1174T>C	p.(Tyr392His)	0.0000	0.0006	-	-	DCM	C65	Deleterious	NA	polymorphism	83	probably damaging	0.0005	NA	156	0.33	NA	NA	V051
SIDS041	F	4	2	rs185947256	*4GL*	NG_0128655.1	NM_0000282.2		missense	exon	c.3290G>A	p.(Arg1097His)	0.0000	0.0000	-	-	GSD	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0000	0.0000	73	0.36	√	NA	V053
SIDS041	F	4	2	rs139790539	*SYNE1*	NG_0128551	NM_1829612		missense	exon	c.18185C>T	p.(Ser6062Leu)	0.0010	0.0000	-	-	EDMD	C0	Deleterious	good	disease-causing	145	possibly damaging	0.0000	0.0000	54	0.46	NA	NA	V052
SIDS041	F	4	2	rs200383755	*GATA5*	NA	NM_0804734		missense	exon	c.56C>G	p.(Ser19Trp)	0.0000	0.0000	Bicuspid aortic valve	DM?	Afib	C0	Deleterious	unknown	disease-causing	177	probably damaging	0.0280	0.0010	87	0.41	NA	NA	V051
SIDS041	F	4	2	rs367825691	*NOTCH1*	NG_0074581	NM_0176173		missense	exon	c.839A>G	p.(Asn280Ser)	0.0000	0.0000	-	-	AOVD	C45	Deleterious	bad	disease-causing	46	probably damaging	0.0015	NA	185	0.48	NA	NA	V051
SIDS044	M	3	2	rs137953195	*ELN*	NG_0092611	NM_0012789391		missense	exon	c.3286>A	p.(Ala1107Thr)	0.0000	0.0000	-	-	connective tissue?	C0	Tolerated	NA	disease-causing	58	probably damaging	0.0000	0.0010	95	0.49	NA	NA	V051
SIDS044	M	3	2	rs376263577	*ETFDH*	NG_0070782	NM_0044533		missense	exon	c.3586>C	p.(Asp1196His)	0.0000	0.0000	-	-	GA	C0	Deleterious	NA	NA	81	possibly damaging	0.0000	0.0000	67	0.34	NA	NA	V051
SIDS044	M	3	2	~	*MYH6*	NG_0234441	NM_0024713		missense	exon	c.2819C>A	p.(Ala940Asp)	NA	NA	-	-	HCM	C65	Deleterious	bad	disease-causing	126	probably damaging	NA	NA	183	0.47	NA	0.0000	V051

Supp. Data Table S4 VUS4-VUS0 in our SIDS cohort (part 2).

Case	Gender	Age (mo)	SIDS category	rs-Nr.	Gene	HGVS RefSeq-Nr.	HGVS genomic RefSeq-Nr.	Coding Effect	Location	cDNA	Protein change	dbSNP ALL MAF	espEA MAF	Human (HGMD)-Phenotype	Gene Mutation	Database	HGMD Variant class	associated disease	AGVGD	SIFT	MAPP	MutationTaster	Grantham distance	polyphen2	EA EURMAF (non-finnish)	1000 Genomes EURMAF	Coverage	Alternative Allele	Sanger confirmed	in-house database	subclass[a] based on scoring scheme
SIDS045	M	5	1	rs369588692	*SCN1B*	NG_013359.1	NM_199037.3	missense	exon	c.673C>T	p.(Arg225Cys)	0.0000	0.0000	-	-	-	Brs, Afib	CO	Deleterious	bad	disease-causing	142	probably damaging	0.0001	0.0000	117	0.46	NA	NA	VUS4
SIDS046	F	3	2	rs374137476	*DSP*	NG_008803.1	NM_004415.2	missense	exon	c.8531G>T	p.(Gly2844Val)	0.0000	0.0001	-	-	-	ARVC	CO	Deleterious	bad	disease-causing	109	probably damaging	0.0000	NA	71	0.48	NA	NA	VUS2
SIDS046	F	3	2	rs147173048	*SYNE2*	NG_011756.1	NM_182914.2	missense	exon	c.16178C>T	p.(Ala5393Val)	0.0000	0.0000	-	-	-	EDMD	CO	Tolerated	bad	disease-causing	64	possibly damaging	0.0000	0.0000	37	0.46	NA	NA	VUS2
SIDS049	F	3	1	rs587780334	*FKRP*	NG_008898.2	NM_024301.4	missense	exon	c.1027G>C	p.(Gly343Gln)	NA	NA	-	-	-	-	CO	Deleterious	bad	disease-causing	29	probably damaging	0.0000	0.0000	78	0.38	NA	NA	VUS1
SIDS049	F	3	1	rs139203363	*SLC22A5*	NG_008898.2.1	NM_0030603	missense	exon	c.34G>A	p.(Gly12Ser)	0.0010	0.0016	systemic primary carnitine deficiency	DM	SPCD	CO	Deleterious	good	NA	56	probably damaging	0.0007	0.0010	141	0.48	NA	V	0.0000	VUS4
SIDS050	M	2	1	~	*ACTN2*	NG_009081.1	NM_0011032	nonsense	exon	c.2671G>T	p.(Glu891*)	NA	0.0000	-	-	DCM	NA	NA	NA	NA	disease-causing	NA	NA	NA	0.0005	0.0010	41	0.44	V	0.0000	VUS4
SIDS050	M	2	1	rs199732064	*TRPM7*	NG_021363.1	NM_017672.4	missense	exon	c.4084C>T	p.(Pro1362Ser)	0.0000	0.0009	-	-	DCM	CO	Deleterious	bad	disease-causing	74	possibly damaging	0.0005	0.0010	127	0.46	NA	NA	VUS3
SIDS050	M	2	1	rs35912339	*MYLK*	NG_029111.1	NM_0530253	missense	exon	c.1007C>T	p.(Pro336Leu)	0.0000	0.0013	-	-	HCM	C15	Deleterious	bad	polymorphism	98	possibly damaging	0.0017	0.0020	32	0.5	NA	NA	VUS1
SIDS050	M	2	1	rs147146713	*TGFBR1*	NG_007461.1	NM_0046122	missense	exon	c.470G>A	p.(Arg157Gln)	0.0000	0.0000	-	-	LDS1	C35	Deleterious	bad	disease-causing	43	benign	0.0004	NA	47	0.4	NA	NA	VUS1
SIDS051	M	2	2	rs140704891	*RANGRF*	NG_028189.1	NM_016492.4	nonsense	exon	c.181G>T	p.(Glu61*)	0.0020	0.0054	Altered function	FP	Afib	NA	NA	NA	NA	disease-causing	NA	NA	0.0053	0.0050	47	0.45	NA	NA	VUS2
SIDS051	M	2	2	rs201148126	*RBM20*	NG_021177.1	NM_001134363.2	missense	exon	c.850G>A	p.(Gly284Arg)	0.0000	0.0006	-	-	DCM	CO	NA	NA	NA	disease-causing	125	probably damaging	0.0000	0.0010	78	0.47	NA	NA	VUS2
SIDS052	F	4	1	rs774949123	*GJD4*	NA	NM_153368.2	Frameshift	exon	c.215dup	p.(Ser73Valfs*180)	0.0000	0.0000	-	-	Afib	NA	NA	NA	NA	NA	NA	NA	NA	0.0000	0.0010	42	0.38	NA	NA	VUS2
SIDS055	M	2	2	rs140322345	*ILK*	NG_029702.1	NM_001014794.2	missense	exon	c.631C>T	p.(Arg211Cys)	0.0010	0.0014	-	-	DCM	C25	Deleterious	NA	NA	disease-causing	180	possibly damaging	0.0008	0.0010	48	0.4	NA	NA	VUS2
SIDS056	M	6	2	rs2838480	*SLC22A5*	NG_008982.1	NM_0030603	missense	exon	c.1451G>T	p.(Gly484Val)	0.0000	0.0000	-	-	SPCD	CO	Deleterious	bad	disease-causing	109	probably damaging	0.0000	NA	19	0.47	NA	NA	VUS2
SIDS056	M	6	2	rs751300489	*ACTA2*	NG_011541.1	NM_001141945.1	missense	exon	c.403T>C	p.(Tyr135His)	0.0000	NA	Thoracic aortic aneurysms	DM	TAAD	C65	Deleterious	bad	disease-causing	83	probably damaging	0.0000	NA	41	0.46	NA	NA	VUS1
SIDS056	M	6	2	rs748609148	*DCHS1*	NG_033858.1	NM_0037372	missense	exon	c.8399C>T	p.(Ser2800Leu)	NA	NA	-	-	MVP	CO	Deleterious	bad	polymorphism	145	probably damaging	0.0000	0.0000	28	0.46	NA	NA	VUS1
SIDS057	M	3	1	rs138714806	*HCN4*	NG_009063.1	NM_0054772	missense	exon	c.1708G>C	p.(Ser568Thr)	0.0000	0.0010	-	-	Brs	CO	Deleterious	bad	disease-causing	58	benign	0.0000	0.0010	42	0.38	NA	NA	VUS2
SIDS060	M	4	2	rs570425717	*PTPN3*	NA	NM_0028293	missense	exon	c.2329G>A	p.(Glu777Lys)	NA	NA	-	-	schizophrenia	CO	Deleterious	bad	disease-causing	119	possibly damaging	0.0000	0.0000	62	0.48	NA	NA	VUS1
SIDS061	M	4	2	rs143767864	*DCHS1*	NG_033858.1	NM_0037372	missense	exon	c.7046G>A	p.(Arg2349Gln)	0.0000	0.0000	-	-	MVP	CO	Deleterious	bad	disease-causing	43	probably damaging	0.0000	0.0010	114	0.46	NA	NA	VUS3
SIDS061	M	4	2	rs142217269	*SCN10A*	NG_031891.2	NM_0065142	missense	exon	c.4586G>A	p.(Cys1523Tyr)	0.0010	0.0027	Brugada syndrome	DM?	Brs	C65	Deleterious	bad	disease-causing	194	probably damaging	0.0018	0.0010	134	0.46	NA	NA	VUS2
SIDS061	M	4	2	rs78727187	*FBN2*	NG_008750.1	NM_0019993	missense	exon	c.4141C>A	p.(His1381Asn)	0.0030	0.0050	-	-	MFS	C65	Deleterious	bad	disease-causing	68	benign	0.0000	0.0010	104	0.39	NA	NA	VUS2
SIDS062	M	3	1	rs139539832	*ABCC9*	NG_028191.1	NM_0056913	missense	exon	c.1859G>A	p.(Arg620Gln)	0.0000	0.0000	-	-	DCM	CO	Deleterious	NA	NA	43	possibly damaging	0.0000	0.0010	34	0.32	NA	NA	VUS1
SIDS062	M	3	1	~	*NOTCH1*	NG_007458.1	NM_0176173	missense	exon	c.4697C>T	p.(Pro1566Leu)	NA	NA	-	-	AOVD	C65	Deleterious	bad	disease-causing	98	probably damaging	NA	NA	31	0.45	NA	0.0000	VUS1
SIDS063	F	3	1	~	*COL5A2*	NG_011799.1	NM_0003933	missense	exon	c.2459C>G	p.(Pro820Arg)	NA	NA	-	-	HCM	CO	Deleterious	bad	polymorphism	98	probably damaging	NA	NA	19	0.21	NA	0.0000	VUS1
SIDS064	F	3	1	rs372607729	*SGCD*	NG_032120.1	NM_0038033	missense	exon	c.761C>A	p.(Thr254Lys)	0.0000	0.0000	-	-	DCM	CO	Tolerated	good	disease-causing	78	benign	0.0000	0.0000	128	0.4	NA	NA	VUS1
SIDS064	F	3	1	rs147896322	*CACNA1C*	NG_008801.2	NM_0086932	missense	exon	c.5263G>A	p.(Val1755Ile)	0.0000	0.0001	-	-	Brs	CO	Tolerated	NA	disease-causing	29	probably damaging	0.0007	0.0010	70	0.46	NA	NA	VUS1
SIDS066	M	4	2	rs147896322	*CACNA1C*	NG_008801.2	NM_1994603	missense	exon	c.5263G>A	p.(Val1755Ile)	0.0000	0.0001	-	-	LQT5	C65	Deleterious	bad	disease-causing	152	probably damaging	0.0014	0.0030	23	0.22	NA	NA	VUS2
SIDS069	M	3	2	rs200893930	*NOTCH1*	NG_007458.1	NM_0176173	missense	exon	c.7645C>T	p.(Arg2549Cys)	0.0000	0.0001	-	-	DCM	C25	Deleterious	bad	disease-causing	180	possibly damaging	0.0000	0.0000	113	0.26	V	NA	VUS4
SIDS073	M	3	2	rs200473206	*DSP*	NG_008803.1	NM_004415.2	splice site	splice site	c.273+5G>A	p.(?)	0.0000	0.0005	ARVC	DM	ARVC	NA	NA	NA	NA	NA	NA	NA	0.0005	NA	50	0.4	NA	NA	VUS3
SIDS075	F	3	2	rs121434557	*GJA5*	NG_009369.2	NM_0052666	missense	exon	c.286G>T	p.(Ala96Ser)	0.0000	0.0000	Atrial fibrillation	DM	Afib	C65	Deleterious	NA	NA	disease-causing	99	probably damaging	0.0000	0.0000	239	0.45	NA	NA	VUS3
SIDS075	F	3	2	rs149272010	*SYNE1*	NG_012855.1	NM_182961.2	missense	exon	c.19534G>A	p.(Ala6512Thr)	NA	0.0000	-	-	EDMD	C55	Deleterious	bad	NA	58	probably damaging	0.0000	0.0000	71	0.41	NA	NA	VUS3
SIDS075	F	3	2	rs398123072	*ACADM*	NG_007045.2	NM_001286043.1	missense	exon	c.157C>T	p.(Arg53Cys)	0.0000	0.0000	MCAD deficiency	DM	MCAD	C25	Deleterious	NA	disease-causing	180	probably damaging	0.0000	NA	31	0.48	NA	NA	VUS2
SIDS075	F	3	2	rs139045510	*AKAP9*	NG_011623.1	NM_0057514	missense	exon	c.10664A>T	p.(Asp3555Val)	0.0010	0.0012	-	-	LQT5	C65	Deleterious	bad	disease-causing	152	probably damaging	0.0014	0.0030	23	0.22	NA	NA	VUS2
SIDS075	F	3	2	rs374028679	*NEXN*	NG_016625.1	NM_1445733	missense	exon	c.616G>C	p.(Glu206Gln)	0.0000	0.0000	-	-	DCM	C25	Deleterious	bad	polymorphism	29	possibly damaging	0.0000	0.0000	35	0.49	NA	NA	VUS2
SIDS075	F	3	2	rs768824318	*MYLK*	NG_029111.1	NM_0530253	missense	exon	c.2776C>T	p.(Arg926Cys)	0.0000	NA	-	-	HCM	C65	Deleterious	bad	disease-causing	180	probably damaging	0.0000	0.0000	NA	0.44	NA	NA	VUS1
SIDS080	F	0	2	rs104894565	*GJB6*	NG_011808.1	NM_0001513	missense	exon	c.113A>T	p.(Asp38Val)	0.0000	0.0070	Glycogen storage disease 1a	DM	GSD	CO	Deleterious	NA	disease-causing	152	probably damaging	0.0000	0.0000	33	0.48	NA	NA	VUS2
SIDS080	F	0	2	rs151090729	*SCN10A*	NG_031891.2	NM_0065142	missense	exon	c.4984G>A	p.(Gly1662Ser)	0.0000	0.0019	Small fibre neuropathy	DM	Brs	C55	Deleterious	good	disease-causing	56	probably damaging	0.0007	0.0010	133	0.48	NA	NA	VUS2
SIDS080	F	0	2	rs146389405	*SYNE1*	NG_012855.1	NM_182961.2	missense	exon	c.26299A>G	p.(Met8767Val)	0.0000	0.0000	-	-	EDMD	CO	Deleterious	bad	NA	21	possibly damaging	0.0000	0.0000	98	0.38	NA	NA	VUS2
SIDS084	M	9	2	rs150955173	*SYNE2*	NG_011756.1	NM_182914.2	missense	exon	c.19415C>T	p.(Ser6472Leu)	0.0000	0.0020	-	-	EDMD	CO	Deleterious	bad	disease-causing	145	probably damaging	0.0030	0.0000	161	0.46	NA	NA	VUS2
SIDS084	M	9	2	rs377192523	*DCHS1*	NG_033858.1	NM_0037372	missense	exon	c.8896A>G	p.(Gln2987Arg)	0.0000	0.0000	-	-	MVP	CO	Deleterious	bad	disease-causing	43	probably damaging	0.0000	NA	41	0.49	NA	NA	VUS1
SIDS085	F	2	2	rs148791938	*DCHS1*	NG_033858.1	NM_0037372	missense	exon	c.5503C>T	p.(Leu1835Phe)	0.0000	0.0000	-	-	MVP	CO	Tolerated	good	disease-causing	22	probably damaging	0.0010	0.0010	75	0.49	NA	NA	VUS1
SIDS087	M	4	1	~	*MYLK*	NG_029111.1	NM_0530253	missense	exon	c.1490C>T	p.(Ser497Phe)	NA	0.0000	-	-	HCM	C65	Deleterious	bad	disease-causing	155	probably damaging	NA	NA	25	0.28	V	0.0000	VUS3
SIDS087	M	4	1	rs199654211	*NOTCH1*	NG_007458.1	NM_0176173	missense	exon	c.3113A>G	p.(Asn1045Ser)	0.0000	0.0200	-	-	AOVD	C15	Deleterious	bad	disease-causing	46	benign	0.0000	0.0000	56	0.25	NA	NA	VUS1
SIDS089	F	5	1	rs199800922	*MYH11*	NG_009299.1	NM_001040114.1	missense	exon	c.3624C>G	p.(His1208Gln)	0.0000	0.0001	-	-	HCM, TAA	C15	Deleterious	bad	disease-causing	24	possibly damaging	0.0000	0.0010	124	0.45	NA	NA	VUS3
SIDS089	F	5	1	rs562115547	*AKAP9*	NG_009006.2	NM_0011484	missense	exon	c.7396C>T	p.(Arg2466Cys)	0.0000	NA	-	-	LQT5	C65	Deleterious	bad	disease-causing	180	possibly damaging	0.0000	0.0000	61	0.48	NA	NA	VUS2
SIDS089	F	5	1	rs138693624	*SYNE1*	NG_012855.1	NM_182961.2	missense	exon	c.10022T>C	p.(Val3341Ala)	0.0060	0.0000	-	-	EDMD	C25	Deleterious	good	NA	64	benign	0.0000	0.0000	53	0.36	NA	NA	VUS1
SIDS091	M	2	2	rs200899037	*CASQ2*	NG_008802.1	NM_0012323	missense	exon	c.1052A>G	p.(Asp351Gly)	0.0000	0.0000	-	-	CPVT	CO	Deleterious	bad	disease-causing	94	probably damaging	0.0000	0.0010	58	0.35	V	NA	VUS3
SIDS091	M	2	2	rs201104206	*MYOM1*	NG_032120.1	NM_0038033	missense	exon	c.1978C>G	p.(Pro660Ala)	0.0000	0.0016	-	-	HCM	CO	Deleterious	bad	disease-causing	27	possibly damaging	0.0030	0.0020	32	0.47	NA	NA	VUS1
SIDS092	F	2	1	rs127204457	*KCNQ1*	NG_008935.1	NM_0002182	missense	exon	c.1179G>T	p.(Lys393Asn)	0.0000	0.0008	LQT5	DM	LQT5	C35	Deleterious	bad	disease-causing	94	possibly damaging	0.0012	0.0040	86	0.47	NA	NA	VUS2
SIDS092	F	2	1	rs754717867	*ANKRD1*	NG_032277.1	NM_0143912	missense	exon	c.544C>T	p.(Arg182Cys)	0.0000	NA	-	-	HCM	C55	Deleterious	bad	disease-causing	180	probably damaging	0.0000	NA	48	0.5	NA	NA	VUS1
SIDS093	F	8	2	rs2234916	*KCNE2*	NG_008804.1	NM_1722011	missense	exon	c.22A>G	p.(Thr8Ala)	0.0020	NA	Altered function	DP	LQT5	C55	Deleterious	bad	disease-causing	58	disease-causing	0.0054	0.0000	143	0.43	NA	NA	VUS1
SIDS096	F	2	1	rs71352737	*TRPM4*	NG_027551.1	NM_0176363	nonsense	exon	c.1575G>A	p.(Trp525*)	0.0010	0.0011	-	-	Brs	NA	NA	NA	NA	disease-causing	NA	NA	0.0018	0.0010	63	0.4	NA	NA	VUS4

Supp. Data Table S4 VUS4-VUS0 in our SIDS cohort (part 3).

Case	Gender	Age (mo)	SIDS category	rs-Nr.	Gene	HGVS RefSeq-Nr.	HGVS genomic-Nr.	HGVS Refseq-Nr.	Coding Effect	Location	c.DNA	Protein change	dbSNP ALUMAF	espEAMAF	Human Gene Mutation (HGMD)-Phenotype	Database	HGMD Variant class	associated disease	AGVGD	SIFT	MAPP	MutationTaster	Grantham distance	polyphen2	ExAC EURMAF (non-finnish)	1000 Genomes EURMAF	Coverage	Alternative Allele	Sanger confirmed	in-house database	subclass based on scoring scheme
SIDS097	F	2	1	rs200286058	*ABCC8*	NG_008867.1	NM_001287174.1	missense	exon	c.2152G>A	p.(Gly718Ser)	0.0000	0.0020	-	-	-	IVF	C55	Deleterious	NA	disease-causing	56	probably damaging	0.0000	0.0000	87	0.46	NA	NA	VUS2
SIDS097	F	2	1	rs147589613	*COL5A1*	NG_008030.1	NM_000093.4	missense	exon	c.341C>A	p.(Ala114Asp)	0.0000	0.0010	-	-	-	EDS	C0	Deleterious	bad	NA	126	possibly damaging	0.0000	0.0000	117	0.44	NA	NA	VUS2
SIDS097	F	2	1	rs142206385	*SYNE2*	NG_011756.1	NM_182914.2	missense	exon	c.15446G>A	p.(Arg5149His)	0.0010	0.0000	-	-	-	EDMD	C0	Tolerated	NA	disease-causing	29	possibly damaging	0.0000	0.0010	79	0.48	NA	NA	VUS1
SIDS098	M	11	1	rs201082652	*SLC22A5*	NG_008982.1	NM_003060.3	missense	exon	c.364G>T	p.(Asp122Tyr)	0.0000	0.0003	systemic primary carnitine deficiency	DM	5PCD	C0	Deleterious	good	NA	160	probably damaging	0.0005	0.0000	64	0.47	NA	NA	VUS2
SIDS098	M	11	1	rs35156360	*MYLK*	NG_029111.1	NM_053025.3	missense	exon	c.1327C>T	p.(Pro443Ser)	0.0060	0.0205	-	-	-	HCM	C65	Deleterious	bad	disease-causing	74	probably damaging	0.0000	0.0130	44	0.45	NA	NA	VUS1
SIDS100	F	4	2	rs373347787	*SCN10A*	NG_031891.2	NM_006514.2	missense	exon	c.4780C>T	p.(Arg1594Cys)	0.0000	0.0000	-	-	-	BrS	G65	Deleterious	bad	disease-causing	180	probably damaging	0.0000	NA	100	0.45	NA	NA	VUS3
SIDS100	F	4	2	rs371316167	*ACADVL*	NG_007975.1	NM_001270447.1	missense	exon	c.1603C>T	p.(Arg535Trp)	0.0000	0.0000	-	-	-	MCAD	C0	Deleterious	good	disease-causing	101	probably damaging	0.0000	0.0000	1.52	0.49	NA	NA	VUS2
SIDS100	F	4	2	rs143842011	*SYNE1*	NG_012855.1	NM_182961.2	missense	exon	c.23659G>A	p.(Val7887Met)	0.0000	0.0000	-	-	-	EDMD	C0	Deleterious	bad	NA	21	probably damaging	0.0000	0.0000	62	0.47	NA	NA	VUS2
SIDS101	M	6	2	rs200899037	*CASQ2*	NG_008802.1	NM_001232.3	missense	exon	c.1052A>G	p.(Asp351Gly)	0.0000	0.0000	-	-	-	CPVT	C0	Deleterious	bad	disease-causing	94	probably damaging	0.0000	0.0010	50	0.32	√	NA	VUS3
SIDS102	F	9	2	rs77357563	*TBX5*	NG_007373.1	NM_000192.3	missense	exon	c.331G>T	p.(Asp111Tyr)	0.0020	0.0049	Holt-Oram syndrome	DM	ASD	C65	Deleterious	bad	disease-causing	160	probably damaging	0.0040	0.0060	98	0.49	NA	NA	VUS1
SIDS103	M	5	2	rs371564200	*MYBPC3*	NG_007667.1	NM_000256.3	missense	exon	c.1828G>C	p.(Asp610His)	0.0000	0.0000	HCM	DM	HCM, DCM, LVNC	C65	Deleterious	bad	disease-causing	81	probably damaging	0.0000	NA	30	0.5	NA	NA	VUS2
SIDS103	M	5	2	rs36210417	*AHC2*	NG_009006.2	NM_001148.4	missense	exon	c.9854T>C	p.(Ile3285Thr)	0.0050	0.0010	-	-	-	LQTS	C65	Deleterious	bad	disease-causing	89	probably damaging	0.0110	0.0080	1.03	0.49	NA	NA	VUS1
SIDS107	M	3	2	rs144605670	*SYNE2*	NG_011756.1	NM_182914.2	missense	exon	c.15781G>A	p.(Val5261Ile)	0.0010	0.0000	-	-	-	EDMD	C0	Deleterious	bad	polymorphism	29	benign	0.0000	0.0000	53	0.49	NA	NA	VUS1
SIDS111	M	3	2	~	*GUSB*	NG_016197.1	NM_000181.3	missense	exon	c.215G>T	p.(Gly72Val)	NA	0.0000	-	-	-	MPS VII	C0	Deleterious	bad	disease-causing	109	probably damaging	NA	NA	107	0.22	NA	0.0000	VUS2
SIDS111	M	3	2	~	*FBN1*	NG_008805.2	NM_000138.4	missense	exon	c.806T>C	p.(Phe269Ser)	NA	NA	-	-	-	MFS	G65	Deleterious	bad	disease-causing	155	probably damaging	NA	NA	55	0.22	NA	0.0000	VUS1
SIDS112	M	2	2	rs150147262	*COL5A1*	NG_008030.1	NM_000093.3	missense	exon	c.514G>T	p.(Val172Phe)	0.0000	0.0015	-	-	-	EDS	C0	Deleterious	bad	NA	50	benign	0.0000	0.0010	67	0.37	NA	NA	VUS1
SIDS114	M	6	2	rs35831865	*SYNE2*	NG_011756.1	NM_182914.2	missense	exon	c.20117G>A	p.(Arg6706Gln)	0.0050	0.0000	-	-	-	EDMD	C0	Deleterious	bad	disease-causing	43	possibly damaging	0.0000	0.0000	48	0.23	NA	NA	VUS2
SIDS115	F	2	2	rs146748294	*SYNE2*	NG_011756.1	NM_182914.2	missense	exon	c.2804A>G	p.(His935Arg)	0.0010	0.0000	-	-	-	EDMD	C0	Deleterious	bad	disease-causing	29	possibly damaging	0.0000	0.0020	70	0.44	NA	NA	VUS2
SIDS117	M	3	2	~	*DSP*	NG_008803.1	NM_004415.2	missense	exon	c.1577T>C	p.(Ile526Thr)	NA	NA	-	-	-	ARVC	C65	Deleterious	bad	disease-causing	89	probably damaging	NA	NA	23	0.35	NA	0.0000	VUS1
SIDS117	M	3	2	rs140413151	*HADH*	NG_008156.2	NM_001184705.2	missense	exon	c.643C>A	p.(Pro215Thr)	0.0020	0.0030	-	-	-	HHF	C35	Deleterious	bad	disease-causing	38	probably damaging	0.0000	0.0050	35	0.46	NA	NA	VUS1
SIDS119	F	1	2	~	*TRPM4*	NG_027551.1	NM_017636.3	Frameshift	exon	c.2470dup	p.(Ala836Serfs*13)	NA	0.0000	-	-	-	BrS	NA	NA	NA	NA	NA	NA	NA	57	0.41	NA	0.0000	VUS3
SIDS120	M	3	2	rs121913013	*DSG2*	NG_007072.3	NM_001943.3	missense	exon	c.1666G>A	p.(Val556Met)	0.0000	0.0035	ARVC	DM	ARVC	C0	Deleterious	good	disease-causing	21	probably damaging	0.0006	0.0010	26	0.42	NA	NA	VUS1
SIDS120	M	3	2	rs77357563	*TBX5*	NG_007373.1	NM_000192.3	missense	exon	c.331G>T	p.(Asp111Tyr)	0.0020	0.0049	Holt-Oram syndrome	DM	ASD	G65	Deleterious	bad	disease-causing	160	probably damaging	0.0040	0.0060	98	0.49	NA	NA	VUS1
SIDS120	M	3	2	rs749485028	*TGFB3*	NG_011715.1	NM_003239.2	missense	exon	c.1054C>T	p.(Arg352Cys)	NA	NA	-	-	-	ARVC	C0	Deleterious	bad	NA	180	probably damaging	0.0000	0.0000	43	0.35	NA	NA	VUS1
SIDS129	M	2	2	rs142191737	*LMNA*	NG_008692.2	NM_170707.2	missense	exon	c.1634G>A	p.(Arg545His)	0.0000	0.0004	Cardiac disease	DM	DCM	C25	Deleterious	bad	disease-causing	29	benign	0.0003	0.0010	53	0.47	NA	NA	VUS2
SIDS130	M	8	2	rs13947361	*SCN5A*	NG_008934.1	NM_001099404.1	missense	exon	c.4366G>A	p.(Val1456Met)	0.0000	0.0000	Brugada syndrome	-	BrS, HCM, LQTS	C15	Deleterious	bad	disease-causing	21	probably damaging	0.0000	NA	36	0.44	NA	NA	VUS1
SIDS131	F	2	2	rs368054375	*SCN3B*	NG_016283.1	NM_018400.3	missense	exon	c.415C>T	p.(Arg139Trp)	0.0000	0.0001	-	-	BrS	C35	Deleterious	bad	disease-causing	101	probably damaging	0.0001	NA	71	0.48	NA	NA	VUS2
SIDS133	F	10	2	rs587780334	*FKRP*	NG_008898.2	NM_024301.4	missense	exon	c.1027G>C	p.(Glu343Gln)	NA	NA	-	-	CMD	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0000	0.0000	145	0.25	NA	NA	VUS1
SIDS140	M	4	2	rs187029309	*FLNA*	NG_011506.1	NM_001110556.1	missense	exon	c.5972C>T	p.(Ser1991Leu)	0.0020	0.0035	-	-	heterotopia	G65	Deleterious	bad	disease-causing	145	probably damaging	0.0030	0.0040	37	homozygote	NA	NA	VUS1
SIDS140	M	4	2	rs77357563	*TBX5*	NG_007373.1	NM_000192.3	missense	exon	c.331G>T	p.(Asp111Tyr)	0.0020	0.0049	Holt-Oram syndrome	DM	ASD	C65	Deleterious	bad	disease-causing	160	probably damaging	0.0040	0.0060	98	0.49	NA	NA	VUS1
SIDS142	M	3	2	rs45465995	*SCN5A*	NG_008934.1	NM_001099404.1	missense	exon	c.5689C>T	p.(Arg1897Trp)	0.0020	0.0004	LQTS	DM	BrS, HCM, LQTS	G65	Deleterious	bad	disease-causing	101	probably damaging	0.0000	0.0020	99	0.49	NA	NA	VUS4
SIDS142	M	3	2	rs140148322	*FHL2*	NG_008844.2	NM_001450.3	missense	exon	c.337C>T	p.(Arg113Cys)	0.0000	0.0005	-	-	DCM	G65	Deleterious	bad	disease-causing	180	probably damaging	0.0005	NA	30	0.27	√	NA	VUS3
SIDS142	M	3	2	rs138504021	*NOTCH1*	NG_007458.1	NM_017617.3	missense	exon	c.1862G>A	p.(Arg621His)	0.0010	0.0010	-	-	AOVD	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0019	0.0030	34	0.5	NA	NA	VUS1
SIDS142	M	3	2	rs144860423	*SNTA1*	NG_011622.1	NM_003098.2	missense	exon	c.566C>T	p.(Ser189Leu)	0.0000	0.0000	-	-	LQTS	C0	Tolerated	good	disease-causing	145	probably damaging	0.0000	0.0100	42	0.38	NA	NA	VUS1
SIDS143	M	6	2	rs369255950	*CACNA1C*	NG_008801.2	NM_199460.3	missense	exon	c.4876G>A	p.(Asp1626Asn)	0.0000	0.0000	-	-	BrS	C0	Tolerated	NA	disease-causing	43	probably damaging	0.0000	0.0000	123	0.2	NA	NA	VUS1
SIDS143	M	6	2	rs112402535	*DCHS1*	NG_033838.1	NM_003737.2	missense	exon	c.3796>A	p.(Val1266Ile)	0.0010	0.0070	-	-	MVP	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0070	0.0010	78	0.2	NA	NA	VUS1
SIDS148	M	5	2	rs148146011	*AKAP9*	NG_011623.1	NM_005751.4	missense	exon	c.4826G>A	p.(Arg1609Lys)	0.0000	0.0000	-	-	LQTS	C25	Deleterious	good	polymorphism	26	benign	0.0000	0.0010	227	0.44	NA	NA	VUS1
SIDS148	M	5	2	rs151021935	*AKAP9*	NG_011623.1	NM_005751.4	missense	exon	c.5369T>G	p.(Val1790Gly)	0.0000	0.0000	-	-	LQTS	C0	Tolerated	good	disease-causing	109	benign	0.0010	0.0000	23	0.43	NA	NA	VUS1
SIDS148	M	5	2	rs56208331	*GATA4*	NG_008177.1	NM_002052.3	missense	exon	c.1273G>A	p.(Asp425Asn)	0.0000	0.0000	Congenital heart defects	DM	ASD	C0	Tolerated	good	disease-causing	23	probably damaging	0.0003	0.0000	131	0.37	NA	NA	VUS1
SIDS149	M	2	2	rs375218723	*DES*	NG_008043.1	NM_001927.3	missense	exon	c.1123C>T	p.(Arg375Trp)	0.0000	0.0000	-	-	DCM	G65	Deleterious	bad	disease-causing	101	probably damaging	0.0000	NA	86	0.5	NA	NA	VUS2
SIDS149	M	2	2	rs142004123	*SOS1*	NG_007530.1	NM_005633.3	missense	exon	c.2371C>A	p.(Leu791Ile)	0.0000	0.0010	-	-	noonan syndrome	C0	Deleterious	bad	disease-causing	5	possibly damaging	0.0010	0.0010	54	0.48	NA	NA	VUS2
SIDS149	M	2	2	rs772783444	*TRDN*	NG_030438.1	NM_006073.3	missense	exon	c.812G>C	p.(Arg271Pro)	NA	NA	-	-	CPVT	C0	Deleterious	NA	disease-causing	103	probably damaging	0.0000	0.0000	39	0.31	NA	NA	VUS1
SIDS150	M	3	2	rs5762361	*LMNA*	NG_008692.2	NM_170707.2	missense	exon	c.1583C>T	p.(Thr528Met)	NA	0.0000	Hutchinson-Gilford progeria syndrome	DM	DCM, ARVC	G65	Deleterious	bad	disease-causing	81	probably damaging	NA	0.0000	38	0.29	NA	NA	VUS2
SIDS150	M	3	2	rs199598944	*CPT1A*	NG_011801.1	NM_001876.3	missense	exon	c.518G>A	p.(Arg173His)	0.0000	0.0000	-	-	5PCD	C0	Deleterious	good	disease-causing	29	benign	0.0000	0.0000	68	0.43	NA	NA	VUS1
SIDS150	M	3	2	rs201019894	*HADH*	NG_008156.2	NM_001184705.2	missense	exon	c.714C>G	p.(Phe238Leu)	0.0000	0.0008	-	-	HHF	C0	Deleterious	NA	disease-causing	22	NA	0.0000	0.0000	130	0.43	NA	NA	VUS1
SIDS150	M	3	2	rs76757102	*MYOZ2*	NG_029747.1	NM_016599.4	missense	exon	c.29A>C	p.(Gln10Pro)	0.0000	0.0001	-	-	HCM	C0	Deleterious	NA	polymorphism	76	possibly damaging	0.0000	0.0000	269	0.49	NA	NA	VUS1
SIDS150	M	3	2	rs113542007	*ZNF365*	NG_021209.1	NM_199451.2	missense	exon	c.1109C>A	p.(Thr370Lys)	NA	0.0000	-	-	others	C0	Tolerated	NA	disease-causing	78	benign	0.0000	0.0010	93	0.37	NA	NA	VUS1
SIDS156	F	2	2	rs139170018	*SYNE1*	NG_012855.1	NM_182961.2	missense	exon	c.15337G>A	p.(Val5113Ile)	0.0000	0.0012	-	-	EDMD	C25	Deleterious	bad	disease-causing	29	probably damaging	0.0010	0.0000	81	0.41	NA	NA	VUS3
SIDS156	F	2	2	rs16833270	*RYR2*	NG_008799.2	NM_001035.2	missense	exon	c.15196G>A	p.(Val5070Ile)	0.0100	0.0004	-	-	CPVT	C25	Deleterious	bad	polymorphism	29	probably damaging	0.0004	0.0000	64	0.33	NA	NA	VUS2
SIDS159	M	2	2	rs150955173	*SYNE2*	NG_011756.1	NM_182914.2	missense	exon	c.19415C>T	p.(Ser6472Leu)	0.0000	0.0020	-	-	EDMD	C0	Deleterious	bad	disease-causing	145	probably damaging	0.0030	0.0000	141	0.48	NA	NA	VUS2
SIDS159	M	2	2	rs138504021	*NOTCH1*	NG_007458.1	NM_017617.3	missense	exon	c.1862G>A	p.(Arg621His)	0.0010	0.0010	-	-	AOVD	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0019	0.0030	54	0.39	NA	NA	VUS1
SIDS161	M	1	2	rs377061868	*TPM1*	NG_007557.1	NM_000366.5	splice site	exon	c.375-5T>C	p.(?)	0.0000	0.0005	DCM	DM?	DCM	NA	NA	NA	NA	NA	NA	NA	0.0004	NA	151	0.45	NA	NA	VUS3
SIDS161	M	1	2	rs371581038	*MYH11*	NG_009299.1	NM_001040114.1	missense	exon	c.5413C>T	p.(Arg1805Trp)	0.0000	0.0000	-	-	HCM, TAA	C35	Deleterious	bad	polymorphism	32	probably damaging	0.0000	NA	86	0.34	NA	NA	VUS1

Supp. Data Table S4 VUS4-VUS0 in our SIDS cohort (part 4).

Case	Gender	Age (mo)	SIDS category	rs-Nr.	Gene	HGVS RefSeq-Nr.	genomic	HGVS Refseq-Nr.	Coding Effect	Location	cDNA	Protein change	dbSNP ALUMAF	espEAMAF	Human [HGMD]-Phenotype	Gene Mutation	Database	HGMD Variant class	associated disease	AGVGD	SIFT	MAPP	MutationTaster	Grantham distance	polyphen2	ExAC EURMAF (non-finnish)	1000 Genomes EURMAF	Coverage	Alternative Allele	Sanger confirmed	in-house database	subclass[a] based on scoring scheme
SIDS172	F	8	2	rs111843122	*RYR2*	NG_008799.2:	NM_001035.2	splice site	c.2907-1G>C	p.(?)	0.0000	0.0000	-	-	-	-	CPVT	NA	NA	NA	NA	NA	NA	0.0000	34	0.47	V	NA	VUS3
SIDS172	F	8	2	rs202147520	*MYBPC3*	NG_007667.1:	NM_000256.3	missense	exon	c.3742G>A	p.(Gly1248Arg)	0.0000	0.0000	HCM	DM	HCM, DCM, LVNC17	C65	Deleterious	bad	disease-causing	125	possibly damaging	0.0000	NA	35	0.46	NA	NA	VUS2
SIDS172	F	8	2	rs140704891	*RANGRF*	NG_028189.1:	NM_016492.4	nonsense	exon	c.181G>T	p.(Glu61*)	0.0020	0.0054	Altered function	FP	Afib	NA	NA	NA	disease-causing	NA	NA	0.0053	0.0050	22	0.36	NA	NA	VUS2
SIDS172	F	8	2	rs141488398	*SYNE2*	NG_011756.1:	NM_182914.2	missense	exon	c.14528T>A	p.(Phe4843Tyr)	0.0000	0.0010	-	-	EDMD	C0	Deleterious	bad	disease-causing	22	possibly damaging	0.0000	0.0010	27	0.48	NA	NA	VUS2
SIDS172	F	8	2	rs751739709	*ANKRD11*	NG_023227.1:	NM_014391.2	missense	exon	c.460C>T	p.(Arg154Trp)	0.0000	NA	-	-	HCM	C65	Deleterious	bad	disease-causing	101	probably damaging	0.0000	NA	60	0.45	NA	NA	VUS1
SIDS176	F	3	2	rs764169067	*SYNE1*	NG_012855.1:	NM_182961.2	missense	exon	c.15366G>T	p.(Lys5122Asn)	NA	NA	-	-	EDMD	C65	Deleterious	bad	NA	94	probably damaging	0.0000	0.0000	83	0.31	NA	NA	VUS2
SIDS184	F	4	2	rs770002854	*AKAP9*	NG_011623.1:	NM_005751.4	missense	exon	c.5639G>A	p.(Arg1880His)	NA	NA	-	-	LQTS	C25	Deleterious	bad	disease-causing	29	possibly damaging	0.0000	0.0000	36	0.22	NA	NA	VUS1
SIDS184	F	4	2	rs139547471	*ELN*	NG_009261.1:	NM_001278939.1	missense	exon	c.2246G>A	p.(Gly749Glu)	0.0000	0.0000	-	-	connective tissue?	C0	Deleterious	NA	disease-causing	98	probably damaging	0.0000	NA	30	0.27	NA	NA	VUS1
SIDS194	F	5	2	rs72544141	*ANK2*	NG_009006.2:	NM_001148.4	missense	exon	c.4373A>G	p.(Glu1458Gly)	0.0010	0.0002	LQTS	DM	LQTS	C65	Deleterious	bad	disease-causing	98	possibly damaging	0.0006	0.0010	63	0.44	NA	NA	VUS4
SIDS194	F	5	2	rs587780334	*FKBP*	NG_008898.2:	NM_024301.4	missense	exon	c.1027G>C	p.(Glu343Gln)	NA	NA	-	-	CMD	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0000	0.0000	55	0.44	NA	NA	VUS1
SIDS194	F	5	2	rs149617373	*SYNE2*	NG_011756.1:	NM_182914.2	missense	exon	c.15248A>G	p.(Asp5083Gly)	0.0000	0.0016	-	-	EDMD	C0	Deleterious	bad	polymorphism	94	benign	0.0012	0.0010	122	0.49	NA	NA	VUS1
SIDS202	M	1	1	rs145169006	*LAMP2*	NG_007995.1:	NM_001122606.1	missense	exon	c.661G>A	p.(Gly221Arg)	0.0010	0.0025	DCM	DM?	HCM	C0	Deleterious	bad	disease-causing	125	possibly damaging	0.0017	0.0000	37	homozygote	NA	NA	VUS1
SIDS202	M	1	1	rs200560685	*DSC2*	NG_008208.2:	NM_024422.3	Frameshift	exon	c.2686_2687d	p.(Asn897Lysfs*4)	0.0060	0.0190	ARVC	DM?	ARVC	NA	NA	NA	NA	180	NA	0.0122	0.0100	168	0.45	NA	NA	VUS1
SIDS212	M	1	2	rs35971313	*BMPR1B*	NG_009245.1:	NM_001256793.1	missense	exon	c.761G>A	p.(Arg254His)	0.0000	0.0012	-	-	others	C0	Deleterious	NA	disease-causing	29	possibly damaging	0.0015	0.0000	89	0.48	NA	NA	VUS1
SIDS212	M	1	2	rs759512753	*SYNE1*	NG_012855.1:	NM_182961.2	missense	exon	c.25241C>T	p.(Thr8414Met)	NA	NA	-	-	EDMD	C0	Deleterious	bad	NA	81	probably damaging	0.0000	0.0000	76	0.47	NA	NA	VUS1
SIDS213	M	1	2	rs139203363	*SLC22A5*	NG_008982.1:	NM_003060.3	missense	exon	c.340>A	p(.Gly125er)	0.0010	0.0016	systemic primary carnitine deficiency	DM	SPCD	C0	Deleterious	good	NA	56	probably damaging	0.0007	0.0010	141	0.48	NA	NA	VUS1
SIDS216	M	7	2	rs763089587	*AKAP9*	NG_011623.1:	NM_005751.4	missense	exon	c.4994A>T	p.(Gln1665Leu)	NA	NA	-	-	LQTS	C25	Deleterious	bad	disease-causing	113	benign	0.0000	0.0000	43	0.47	NA	NA	VUS1
SIDS217	M	3	2	rs35912339	*MYLK*	NG_029111.1:	NM_053025.3	missense	exon	c.1007C>T	p.(Pro336Leu)	0.0000	0.0013	-	-	HCM	C15	Deleterious	bad	polymorphism	98	possibly damaging	0.0017	0.0020	39	0.38	NA	NA	VUS1
SIDS219	M	10	2	rs78727187	*FBN2*	NG_008750.1:	NM_001999.3	missense	exon	c.4141C>A	p.(His1381Asn)	0.0030	0.0050	-	-	MFS	C65	Deleterious	bad	disease-causing	68	possibly damaging	0.0000	0.0010	93	0.49	NA	NA	VUS2
SIDS220	M	5	1	rs138797058	*SYNE2*	NG_011756.1:	NM_182914.2	missense	exon	c.15848A>G	p.(Asp5283Gly)	0.0000	0.0000	-	-	EDMD	C0	Deleterious	bad	polymorphism	94	probably damaging	0.0000	0.0010	57	0.47	NA	NA	VUS2
SIDS220	M	5	1	rs587780334	*FKBP*	NG_008898.2:	NM_024301.4	missense	exon	c.1027G>C	p.(Glu343Gln)	NA	NA	-	-	CMD	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0000	0.0000	39	0.46	NA	NA	VUS1
SIDS220	M	5	1	rs140233643	*MYLK2*	NG_012847.1:	NM_033118.3	missense	exon	c.463A>T	p.(Ile155Phe)	0.0000	0.0001	-	-	HCM	C0	Deleterious	good	polymorphism	21	probably damaging	0.0000	0.0000	43	0.4	NA	NA	VUS1
SIDS223	F	2	1	rs148323523	*DCHS1*	NG_033858.1:	NM_003737.2	missense	exon	c.6467T>A	p.(Val2156Glu)	0.0000	0.0006	-	-	MVP	C65	Deleterious	bad	disease-causing	121	possibly damaging	0.0002	0.0000	122	0.48	NA	NA	VUS4
SIDS225	F	3	2	rs141159831	*MYH11*	NG_009299.1:	NM_001040114.1	missense	exon	c.3970C>A	p.(Leu1324Ile)	0.0030	0.0000	-	-	HCM, TAA	C0	Deleterious	bad	disease-causing	5	possibly damaging	0.0000	0.0000	66	0.33	NA	NA	VUS2
SIDS225	F	3	2	rs74120667	*NEB1*	NG_017092.1:	NM_006393.2	missense	exon	c.2057T>C	p.(Val686Ala)	0.0070	0.0000	-	-	DCM	C0	Deleterious	bad	disease-causing	64	benign	0.0000	0.0000	124	0.32	NA	NA	VUS2
SIDS225	F	3	2	rs147173048	*SYNE2*	NG_011756.1:	NM_182914.2	missense	exon	c.16178C>T	p.(Ala5393Val)	0.0000	0.0000	-	-	EDMD	C0	Tolerated	bad	disease-causing	64	possibly damaging	0.0000	0.0000	65	0.25	NA	NA	VUS2
SIDS225	F	3	2	rs587780334	*FKBP*	NG_008898.2:	NM_024301.4	missense	exon	c.1027G>C	p.(Glu343Gln)	NA	NA	-	-	CMD	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0000	0.0000	38	0.34	NA	NA	VUS1
SIDS226	F	2	1	rs151242451	*DMD*	NG_012232.1:	NM_004006.2	missense	exon	c.2261G>T	p.(Gly754Val)	0.0020	0.0000	-	-	DCM	C0	Tolerated	good	disease-causing	109	probably damaging	0.0000	0.0000	126	0.44	NA	NA	VUS2
SIDS226	F	2	1	rs36026860	*SEMA3A*	NG_011489.1:	NM_006080.2	missense	exon	c.1198A>G	p.(Ile400Val)	0.0010	0.0000	Kallmann syndrome	DM	IVF	C0	Deleterious	bad	disease-causing	29	benign	0.0000	0.0000	259	0.48	NA	NA	VUS2
SIDS226	F	2	1	rs150179494	*SYNE1*	NG_012855.1:	NM_182961.2	missense	exon	c.12170C>T	p.(Pro4057Leu)	0.0010	0.0000	-	-	EDMD	C15	Deleterious	bad	NA	98	probably damaging	0.0000	0.0010	83	0.36	NA	NA	VUS2
SIDS226	F	2	1	rs35530544	*ANK2*	NG_009006.2:	NM_001148.4	missense	exon	c.11218C>A	p.(Leu3740Ile)	0.0090	0.0001	Cardiac arrhythmia	DM	LQTS	C0	Tolerated	good	disease-causing	5	possibly damaging	0.0000	0.0010	131	0.47	NA	NA	VUS1
SIDS226	F	2	1	rs142873474	*CTGF*	NG_016131.1:	NM_001901.2	missense	exon	c.645G>A	p.(Met215Ile)	0.0000	0.0000	-	-	cutis laxa	C0	Tolerated	good	disease-causing	10	possibly damaging	0.0000	0.0000	124	0.4	NA	NA	VUS1
SIDS226	F	2	1	rs76927619	*RAB3GAP*	NG_016972.1:	NM_001172435.1	missense	exon	c.669G>T	p.(Leu223Phe)	0.0090	0.0000	-	-	CMRH	C0	Tolerated	good	disease-causing	22	probably damaging	0.0000	0.0000	213	0.5	NA	NA	VUS1
SIDS226	F	2	1	rs35654757	*SYNE1*	NG_012855.1:	NM_182961.2	missense	exon	c.19698G>T	p.(Met6566Ile)	0.0050	0.0000	-	-	EDMD	C0	Deleterious	bad	NA	10	benign	0.0000	0.0000	44	0.41	NA	NA	VUS1
SIDS226	F	2	1	rs144108695	*SYNE2*	NG_011756.1:	NM_182914.2	missense	exon	c.10313G>A	p.(Cys3438Tyr)	0.0000	0.0000	-	-	EDMD	C0	Tolerated	good	polymorphism	194	possibly damaging	0.0000	0.0000	222	0.47	NA	NA	VUS1
SIDS227	M	4	2	rs765792077	*MYH6*	NG_023444.1:	NM_002471.3	missense	exon	c.100C>T	p.(Arg34Cys)	0.0000	NA	-	-	HCM	C55	Deleterious	bad	polymorphism	180	probably damaging	0.0010	0.0000	91	0.35	NA	NA	VUS2
SIDS227	M	4	2	rs143584663	*NEB1*	NG_017092.1:	NM_006393.2	missense	exon	c.2654C>T	p.(Ser885Phe)	0.0010	0.0040	-	-	DCM	C0	Deleterious	bad	disease-causing	155	probably damaging	0.0000	0.0050	48	0.33	NA	NA	VUS1
SIDS227	M	4	2	rs74820467	*SYNE2*	NG_011756.1:	NM_182914.2	missense	exon	c.41686>C	p.(Asp13901His)	NA	NA	-	-	EDMD	C0	Deleterious	bad	polymorphism	81	probably damaging	0.0000	0.0000	76	0.41	NA	NA	VUS1
SIDS234	M	2	2	rs77160178	*HMGCS2*	NG_013348.1:	NM_005518.3	splice site	c.1016+1G>A	p.(?)	0.0000	NA	Mitochondrial HMG-CoA synthase	DM	HMGCS	NA	NA	NA	NA	NA	NA	NA	0.0000	NA	35	0.49	NA	NA	VUS1
SIDS235	F	6	2	rs776254819	*ANK2*	NG_009006.2:	NM_001148.4	missense	exon	c.2296>A	p.(Val77Met)	NA	NA	-	-	LQTS	C0	Deleterious	bad	disease-causing	21	probably damaging	0.0000	0.0000	49	0.47	NA	NA	VUS1
SIDS236	M	2	2	rs77170569	*KCND3*	NG_032011.2:	NM_004980.4	missense	exon	c.1292G>A	p.(Arg431His)	NA	NA	-	-	BrS	C0	Deleterious	bad	disease-causing	29	probably damaging	0.0000	0.0000	22	0.32	NA	NA	VUS1
SIDS236	M	2	2	rs36083134	*TMEM43*	NG_008975.1:	NM_024334.2	missense	exon	c.1096G>A	p.(Ala366Thr)	0.0060	0.0000	-	-	ARVC	C0	Tolerated	good	disease-causing	58	possibly damaging	0.0000	0.0000	60	0.47	NA	NA	VUS1
SIDS237	F	1	2	rs141159831	*MYH11*	NG_009299.1:	NM_001040114.1	missense	exon	c.3970C>A	p.(Leu1324Ile)	0.0030	0.0000	-	-	HCM, TAA	C0	Deleterious	bad	disease-causing	5	possibly damaging	0.0000	0.0000	76	0.39	NA	NA	VUS2
SIDS237	F	1	2	rs74120667	*NEB1*	NG_017092.1:	NM_006393.2	missense	exon	c.2057T>C	p.(Val686Ala)	0.0070	0.0000	-	-	DCM	C0	Deleterious	bad	disease-causing	64	benign	0.0000	0.0000	133	0.29	NA	NA	VUS2
SIDS237	F	1	2	rs147173048	*SYNE2*	NG_011756.1:	NM_182914.2	missense	exon	c.16178C>T	p.(Ala5393Val)	0.0000	0.0000	-	-	EDMD	C0	Tolerated	bad	disease-causing	64	possibly damaging	0.0000	0.0000	32	0.28	NA	NA	VUS2
SIDS238	M	7	1	rs140704891	*RANGRF*	NG_028189.1:	NM_016492.4	nonsense	exon	c.181G>T	p.(Glu61*)	0.0020	0.0054	Altered function	FP	Afib	NA	NA	NA	disease-causing	NA	NA	0.0053	0.0050	55	0.49	NA	NA	VUS2
SIDS239	M	2	1	rs374482089	*SYNE1*	NG_012855.1:	NM_182961.2	missense	exon	c.62686>C	p.(Glu2090Gln)	0.0000	0.0000	-	-	EDMD	C25	Deleterious	bad	NA	29	benign	0.0000	0.0000	26	0.31	NA	NA	VUS2

Abbreviations:
F: female, M: male, mo: months, NA: not available.
DM: disease-causing, DM?: Potentially disease-causing, DP: disease-causing polymorphisms reported to be significant association with diseases and assumed to be functional, FP: in vitro or in vivo functional polymorphism.
Afib: atrial fibrillation, AOVD: aortic valve disease, ARVC: arrhythmogenic right ventricular cardiomyopathy, ASD: aortic septal defect, BrS: Brugada syndrome, CMD: congenital muscular dystrophy, CMRH: cataract-mental retardation-hypogonadism, CPVT: catecholaminergic polymorphic ventricular tachycardia, CVDX: cardiac valvular dysplasia x-linked, DCM: dilated cardiomyopathy, EDMD: Emery-dreifuss muscular dystrophy, EDS: Ehlers-Danlos syndrome, GA: glutaric aciduria, GSD: glycogen storage disease, HCM: hypertrophic cardiomyopathy, HHF: familial hyperinsulinemic hypoglycaemia, HMGCS: HMG-CoA synthase deficiency, IVF: idiopathic ventricular fibrillation, LQTS: long QT syndrome, LVNC: left ventricular noncompaction cardiomyopathy, MCAD: medium-chain-acyl-CoA-dehydrogenase deficiency, MVP: mitral valve prolapse, MFS: marfan syndrome, PAH: pulmonary hypertension, QKTS: outward rectifier potassium channel, RCM: restrictive cardiomyopathy, TAA: familial thoracic aortic aneurysm and aortic dissection.
AGVGD: Grantham variation/Grantham deviation, MAPP: multiple alignment probability prediction, MAPP: multivariate analysis of protein polymorphism prediction, MAFF: minor allele frequency, dbSNP: MAF in general population in NCBI dbSNP, EspEAMAF: MAF in European American population in NHLBI GO Exome Sequencing Project, ExAC EURMAF: MAF in European population in exome aggregation consortium - 1000 Genomes Project EURMAF: MAF in European population in 1000 Genomes Project
"-no dbSNP rs-nr. available.
- no information available.
[a]based on 118 in-house control exons.

2.3 Manuscript III

Exome analysis in 34 sudden unexplained death (SUD) victims mainly identified variants in channelopathy-associated genes

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Abstract

Sudden cardiac death (SCD) is one of the major causes of mortality worldwide, mostly involving coronary artery disease in the elderly. In contrary, sudden death events in young victims often represent the first manifestation of undetected genetic cardiac diseases, which remained without any symptoms during lifetime. Approximately 30% of these sudden death cases have no definite cardiac etiology after a comprehensive medicolegal investigation and are therefore termed as sudden unexplained death (SUD) cases. Advances in high-throughput sequencing approaches have provided an efficient diagnostic tool to identify likely pathogenic variants in cardiovascular disease-associated genes in otherwise autopsy-negative SUD cases. The aim of this study was to genetically investigate a cohort of 34 unexplained death cases by focusing on candidate genes associated with cardiomyopathies and channelopathies. Exome analysis identified potentially disease-causing sequence alterations in 29.4% of the SUD cases. Six (17.6%) individuals had variants with likely functional effects in the channelopathy-associated genes *AKAP9*, *KCNE5*, *RYR2*, and *SEMA3A*. Interestingly, four of these six SUD individuals were younger than 18 years of age. Since the total SUD cohort of this study included five children and adolescents, post-mortem molecular autopsy screening indicates a high diagnostic yield within this age group. Molecular genetic testing represents a valuable approach to uncover the cause of death in some of the SUD victims, however, 70-80% of the cases still remain elusive, emphasising the importance of additional research to better understand the pathological mechanisms leading to a sudden death event.

Introduction

Sudden and unexpected death of a previously healthy infant, adolescent or young adult is a tragic and distressing event for those left behind. The majority of these sudden death cases are caused by undiagnosed genetic cardiac diseases, which can be uncovered by a comprehensive medicolegal investigation, including autopsy[1]. However, up to 30% of all sudden death cases in young individuals between 1 to 40 years of age remain unexplained after standard forensic autopsy, with no definite cardiac etiology identified after gross and microscopic inspection of the heart[2]. These cases are generally designated as autopsy-negative sudden unexplained death (SUD) cases. The exact incidence of SUD in the young remains unknown in most countries, including Switzerland[3].

Many of the SUD cases are considered to be caused by genetic cardiac diseases causing lethal arrhythmias, not detectable during conventional forensic autopsy investigation[4]. Channelopathies primarily affect the heart rhythm and cardiac electrical conduction, including long QT syndrome (LQTS), short QT syndrome (SQTS), catecholaminergic polymorphic ventricular tachycardia

(CPVT), Brugada syndrome (BrS), and idiopathic ventricular fibrillation (IVF)[5]. Cardiomyopathies are characterized by a structural and functional abnormal heart muscle; however, initial slight phenotypic alterations might not be visible at autopsy, making a proper diagnosis difficult[6]. The most common form is hypertrophic cardiomyopathy (HCM), followed by arrhythmogenic right ventricular cardiomyopathy (ARVC), idiopathic dilated cardiomyopathy (DCM), and left ventricular non-compaction cardiomyopathy (LVNC)[7].

In the last years, high-throughput massive parallel sequencing (MPS) approaches had facilitated the genetic investigation of SUD cohorts in a time- and cost-efficient manner[8,9]. A recently published first proof-of-principle case report of a whole-exome sequencing (WES) analysis identified a pathogenic *MYH7* mutation in an otherwise healthy 16-year-old SUD victim, which has been described in familial HCM, sudden death, impaired MHC-β actin-translocating and actin-activated ATPase activity[10]. Subsequent studies in other SUD cohorts described the detection of likely functional variants in genes involved in cardiomyopathies and cardiac channelopathies in 20-35% of autopsy-negative SUD cases[11-15].

The aim of this study was to explore the proportion of cardiovascular disease-associated sequence alterations in a cohort of 34 SUD victims, collected at the Zurich Institute of Forensic Medicine (ZIFM) in Switzerland.

Material and Methods

SUD cohort

Between 2013 and 2015, 34 unrelated sudden unexplained death cases had been autopsied at the Zurich Institute of Forensic Medicine (ZIFM) in Switzerland. Although general inclusion criteria for SUD cases per definition only include individuals younger than 40 years of age, older autopsy-negative sudden death cases were included in our cohort if there had been a suspicion of a cardiac disease in absence of any evidence for myocardial infarction. All SUD victims were examined according to standard forensic procedures, including death scene investigation, a complete autopsy examination with pharmacological-toxicological and histopathological screening, and review of the clinical history. All samples were anonymised and family members were not available for co-segregation analyses.

The cohort was composed of 26 males (mean ± SD, 33.07 ± 12.85 years; range, 1 – 63 years) and 8 females (mean ± SD, 23.62 ± 15.34 years; range = 5 – 50 years) (Table 1). Five SUD victims were younger than 18 years of age, including 3 females (aged 5, 7, and 11 years) and 2 males (aged 1 and 17 years). Most of the SUD individuals had an European origin (85.4%). Additional three cases (8.9%) were Africans (one Afghan, one Nigerian, and one not further specified), and two (5.8%) were Asians (one Chinese and one Indian).

Ethical approval for this study was provided by the local committee in Zurich (KEK-ZH-Nr. 2013-0086), and the study was conducted in full conformance with Swiss laws and regulations.

Tissue collection and DNA extraction

During autopsy, several 1-2cm^3 pieces of the heart, kidney and spleen were shock frozen in liquid nitrogen and afterwards stored at -80°C. In addition, EDTA blood samples were collected and stored at -20°C. DNA extractions of shock frozen heart and kidney tissue of 31 SUD were performed using the QIAmp DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. The DNA of the remaining three individuals was extracted from blood samples using the QIAamp DNA Blood Kit (Qiagen). DNA quantities were determined with a Quantus fluorometer (Promega, Dübendorf, Switzerland).

Exome sequencing and bioinformatics

Exome sequencing and bioinformatics analyses were done as described previously[16]. In brief, SureSelect^{XT} target enrichment and SureSelect^{XT} All Exon V5 + UTR's kits (Agilent Technologies AG, Basel, Switzerland) were used for DNA library preparation and exome capture. DNA libraries were sequenced on an Illumina HiSeq2500 instrument (Illumina Inc., San Diego, USA). Sequences were aligned to the reference genome (GRCh37/hg19) using BWA[17] and samples were required to have at least 80% of the exome covered at ≥ 20x read depth. Variants discovery was performed by means of GATK[18], following the GATK best practices workflow[19].

Data analysis

Data analysis was based on a candidate gene panel of 192 genes, mainly associated with cardiovascular disease-associated genes (Supp. Data Table S1)[16]. Annotation of the vcf-files was performed with the software Alamut Batch version 1.4.2 (Interactive Biosoftware, Rouen, France). Filtering strategy focused on (1) a global minor allele frequency value (MAF) of less than or equal to 0.01 based on NCBI dbSNP, (2) selection of exonic and splice site variants, and (3) the exclusion of synonymous variants (Supp. Data Figure S1). Alamut Visual Version 2.8.1 (Interactive Biosoftware) was used to visualise coverage of variants and to review conservation of the variants across a variety of species. Assessment of pathogenicity was based on a scoring scheme, which evaluates the variant types (null-variants, splice site variants, missense variants), *in silico* protein predictions, and MAF in three European control populations (Supp. Data Table S2 and S3)[12,16,20]. Co-segregation and functional analyses would have been required to classify a variant as pathogenic[20], therefore all sequence alterations were labelled as variants of unknown significance (VUS). DNA variants were numbered according to reference sequences using HGVS nomenclature (http://varnomen.hgvs.org). Identified sequence alterations have been submitted to the Leiden Open Variation Database (Individual IDs: 00103739-00103750) (http:// http://databases.lovd.nl/shared/diseases/05166).

Confirmation and validation of variants

Potential disease-causing variants not reported in the mentioned databases and variants with <50x bidirectional coverage and/or an alternate allele frequency ratio <0.4 were confirmed by standard Sanger sequencing.

Results

Medicolegal investigations

Demographic characteristics of the 34 SUD victims are depicted in Table 1. In our SUD cohort, the most prevalent circumstances of a sudden death event were (1) at rest/sleep (64.7%), followed by (2) sportive exercises (23.5%) such as swimming or hiking and (3) physical exercises during work (11.76%). Symptoms prior to death are unknown in 55.8% of the cases, mainly due to an unwitnessed death event. For some of the other cases, witnesses reported dyspnoea, vomiting, head and stomach pain immediately preceding death.

Autopsy investigations revealed an increased heart size according to Zeek[21] in 58.6% of the adult SUD cases, mostly in males (94.5%). In addition, 64.7% of these adults had a BMI value larger than 25 (mean ± SD, 30.41 ± 2.55; range = 27.8 – 35.5, n = 11). Furthermore, morphological and histological examinations of the heart revealed a range of nonspecific, structural abnormalities, including fibrosis, myocyte disarray and fat replacement. However, none of these changes were characteristic for a specific cardiac disease. Post-mortem pharmacological-toxicological screening tests specified positive screening results in 12 (32.4%) SUD victims, but in none of them at toxic levels. Six (16.2%) individuals were positive for therapeutic drugs in accordance with their medical history, five (13.5%) were positive for alcohol, and four (10.8%) for illegal drugs.

A medical history was reported in almost half of the SUD cases (44.1%) and included asthma, atrial fibrillation, epilepsy, hypertension, Lenegre-Lev syndrome, renal insufficiency, and Wolff-Parkinson-White syndrome. A family history of SCD was documented in 4 cases (11.7%) and the sudden death event of close relatives occurred in 3 cases before the age of 50. In addition, one SUD individual had a family history of a cardiac disease, namely a LQTS diagnosis in the son.

Exome sequencing results

Whole-exome sequencing was successfully completed for all 34 SUD cases. Approximately 40 000 variants per case were obtained within the total WES data. By focusing on 192 genes of interests, we detected an average of 1697 ± 658 variants per case. Based on our scoring scheme, eleven missense variants were categorized as variants with probably pathogenic effects detected in ten (29.4%) individuals of our SUD cohort (Table 2). Additional details of the variants are available in Supplementary Table S4. A seven-year old female carried two likely disease-causing

sequence alterations in *RYR2* (c.12490C>G, p.(Gln4164Glu)) and *ACAD9* (c.1258C>T, p.(Arg420Cys)). Six (11.7%) SUD victims had variants with likely functional effects in four channelopathy-associated genes; *AKAP9* (c.6134A>G, p.(Asn2045Ser)), *KCNE5* (c.184T>A, p.(Tyr62Asn)), *RYR2* (c.12490C>T, p.(Gln4164); c.11441C>T, p.(Ala3814Val) and c.3380A>G, p.(Glu1127Gly)), and *SEMA3A* (c.196G>A, p.(Arg66Trp)). Two of the variants detected in *RYR2* and the variant in *KCNE5* have not been previously described in databases. Additional three variants (*EFEMP2*, c.554C>T, p.(Arg185His); *FBN2*, c.7296C>A, p.(Gln2432His); and *MYLK*, c.3749C>T, p.(Arg1250His)) were detected in genes associated with connective tissue diseases and one sequence alteration (*BMPR2*, c.2591C>T, p.(Pro864Leu)) in a gene associated with pulmonary arterial hypertension.

Discussion

In the last years, high-throughput massive parallel sequencing approaches demonstrated a promising tool to investigate patients with complex disorders or large cohorts in absence of specific phenotypes in a cost- and time efficient manner[9-12,15,22,23]. Here in this study, we sequenced the exomes of 34 unrelated SUD cases and identified ten (29.4%) individuals with potentially disease-causing variants, mostly in genes associated with channelopathies. These findings are similar to other genetic studies where it has been reported that genetic testing in SUD victims has a yield of near 30%[11-15]. Sequence alterations in cardiac channels are described to cause lethal arrhythmias in absence of any structural changes in the heart and therefore represent one of the most plausible and comprehensible underlying causes of death in SUD cases[24].

Three SUD individuals (8.8%) hosted variants in the *RYR2*-encoding ryanodine receptor/calcium release channel gene, in which sequence alterations have previously been reported to occur in 5-10% of SUD cases[29,30]. Ryanodine receptors are located in the sarcoplasmic/endoplasmic reticulum membrane and are responsible for the release of Ca2+ from intracellular stores during cardiac excitation-contraction coupling[25]. In addition, the receptor is also expressed in the central nervous system and contributes to vesicular transmitter release and postsynaptic dendritic spine function[26]. Gain-of-function mutations in the *RYR2* gene have been described in sudden cardiac death cases linked to sudden unexplained death in epilepsy (SUDEP) victims[27] as well as to CPVT, characterized by dysrhythmia with stress-induced syncope and a 30-50% mortality rate before the age of 30[28].

One of the three heterozygous *RYR2* missense variants found in our SUD cohort was detected in a seven-year old female (BMI 15.4; European origin). In addition to the *RYR2* (p.Gln4164Glu) variant, we identified a second likely disease-causing variant in the acyl-CoA dehydrogenase family member 9 encoding gene *ACAD9*, in which variants are associated with mitochondrial

complex I deficiency characterized with a wide range of clinical signs, including lactic acidosis, cardiomyopathy, pure myopathy and hepatopathy[29]. A second *RYR2* (p.(Ala3814Val)) sequence alteration was identified in an eleven-year old female (BMI 16.2; European origin) who was swimming in a lake, when she suddenly disappeared in the water and drowned. Her medical history was inconspicuous and no sudden death events were reported in her family. The third *RYR2* variant (p.(Glu1127Gly)) was reported in an 27 year-old man (BMI 30.6; 31% enlarged heart according to Zeek; European origin) who was found dead in a dorsal position on the floor of his flat. According to his medical history, an epileptic seizure was documented seven years before his death. Two years later, he suffered a spontaneous atrial fibrillation, which remained without any clinical signs during medical follow-up examinations. Furthermore, a recent knee problem led to a drastic increase of the body weight in the former athletic man. Since variants in *RYR2* were reported with generalized tonic-clonic seizures at rest and exercise-induced arrhythmias[30], a combination of epileptic seizures and/or cardiac arrhythmia of the here identified *RYR2* sequence alterations might have contributed to the early death in these three SUD victims. Additional three variants with likely functional effects in channelopathy-associated genes were identified in two individuals younger than 18 years of age and in a 32-years old man.

The 32-years old man (BMI 24.8; normal heart according to Zeek; African origin) carried a heterozygous missense sequence alteration in the A-kinase anchoring protein *AKAP9* (p.(Asn2045Ser)). He was found dead in his bed, after he mentioned a seizure the evening before. A-kinase-anchoring proteins form macromolecular complexes with voltage-gated potassium channel α-subunits (*KCNQ1*) and its associated β-subunits (*KCNE1*), which are responsible for the slowly activating delayed-rectifier K+ current in the heart[31]. Mutations in *AKAP9* are associated with LQTS and have been described as genetic modifier of congenital LQTS in a South African population[32]. Furthermore, patients with LQTS may develop seizures related to an acute hypoxic-ischemic event associated with a ventricular arrhythmia[33], which could also be the underlying pathophysiological cause in this sudden death victim.

In a five-year old girl (BMI 12.4; African origin), we identified a heterozygous missense variant in the voltage-gated potassium channel subfamily E encoding gene *KCNE5* (p.(Tyr62Asn)). The girl was playing with her siblings, when she suddenly collapsed and died. The girl was healthy, but she had a diagnosis of a slight cognitive developmental disorder. *KCNE5* encodes an auxiliary β-subunit for potassium channels and variants have been described in BrS and idiopathic ventricular fibrillation[34].

A 17-years old male (BMI 23.1; European origin) carried a heterozygous missense variant in the semaphorin 3A encoding gene *SEMA3A* (p.(Arg66Trp)). He went swimming in a lake when he suddenly disappeared in the water and drowned. He had an inconspicuous medical history and no

cardiac death events were reported in his family. Semaphorins are extracellular and membrane associated proteins involved in many different cellular processes, mainly in nervous system development through neural migration and axon guidance, but also in cardiac innervation patterning[35]. In addition, SEMA3A has an amino acid motif that is analogous to hanatoxin, an inhibitor of voltage-gated potassium channels. *SEMA3A* gain-of-function mutations have been described as potential contributor to BrS by disrupting SEMA3A's natural ability to supress the K_v4.3 ion channel thus resulting in an increase in potassium channel current[36]. BrS is associated with ventricular fibrillation and a high risk for sudden cardiac death, predominantly in younger males[37], consequently this *SEMA3A* (p.(Arg66Trp)) variant might have contributed to the sudden death event in this 17-years old adolescent.

Interestingly, four of the six above described cases were younger than 18 years of age. Since our SUD cohort included five children/adolescents, we identified likely disease-causing sequencing alterations in 80% of this age group. This trend towards a higher mutation detection rate in very young SUD individuals has already been observed in other studies[14,24,38], suggesting that especially in children and young adults a molecular autopsy is highly recommended.

Another four variants were detected in genes associated with connective tissue diseases and pulmonary arterial hypertension. Although these variants were reported as likely pathogenic based on in silico protein predictions tools and show rare allelic frequencies in the general population, these sequence alterations most probably did not directly contribute to the sudden death event. Nevertheless, they might act as predisposing risk factors in combination with other variants not yet identified within the exome data.

Due to advances in the genetic field, exome sequencing has become more assessable in recent years. However, the generation of such huge amount of data requires very stringent variant evaluation systems in order to separate false-positive variants from truly disease-causing sequence alterations[20]. Several studies have demonstrated that many variants previously associated with cardiac diseases have no or only minor functional effects and are therefore less likely associated with a dominant form of the disease, still they could act as risk modifier[39-41]. Consequently, functional studies are strongly required and recommended for an evidence-based classification of the pathogenicity of variants identified in genetic SUD studies so far[42].

Despite tremendous efforts to identify underlying pathogenic diseases in SUD cases in the last years, 70-80% of the SUD victims still remain elusive after extensive genetic testing, emphasizing the importance of additional research in other directions to better understand the pathological mechanisms leading to a sudden death event. As an example, gene expression has a significant importance in physiological and pathological mechanism and may yield some new insights into mechanisms of sudden death. A number of microRNAs (miRNAs) have been identified to be

highly expressed in non-diseased cardiac tissue and thus likely play a key role in both normal cardiac maintenance and diseases[43]. Consequently, transcriptome analysis in SUD cases might provide a catalogue of genes that show altered expression profiles in sudden death victims or might identify cardiac disease specific transcription levels of regulatory RNAs.

A limitation of this study is the rather small sample size of our SUD cohort, especially in the group younger than 18 years of age. Furthermore, functional assays are crucial in order to verify the potentially pathogenic role of detected sequence alterations, in particular amino acid substitutions. In addition, family members were not available for co-segregation studies. Such analyses would be necessary to determine the mode of inheritance and to identify other genetic carriers at risk for sudden cardiac death.

In conclusion, post-mortem molecular autopsy investigation in sudden death victims has been demonstrated to be very helpful in discovering underlying pathogenic mechanisms in cardiovascular diseases, mainly in young SUD individuals. However more effort is needed to better understand the pathophysiology leading to sudden death.

Supplementary information

Supplementary information is available at the European Journal of Human Genetics' website.

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Compliance with ethical standards

The authors declare that they have no conflict of interest.

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Table 1 Demographic characterization of the SUD cohort.

Table 1 Demographic characteristics of the SUD cohort.

SUDS case	Gender	Age (years)	Ethnicity	BMI	Heart weight (gr)	Enlarged heart weights according to Zeek[21]	Symptoms prior to death	Event at death	Post-mortem toxicology	Medical history	Family history of SD	Presumed cause of death based on autopsy
SUDS001	M	38	European	22.22	430	27%	Drunken	Sleeping	Alcohol (1.93‰)	No	No	Acute heart failure
SUDS003	M	17	European	23.05	310	Normal	n.s.	Swimming	Negative	Lenegre-Lev syndrome	No	Combination of drowning and arrhythmia
SUDS005	M	24	Chinese	21.06	360	Normal	Dyspnea	Sleeping	Negative	Head pain	Unknown	Arrhythmia
SUDS006	F	7	European	15.45	122	n.s.	None	Playing	Negative	Unknown	No	Arrhythmia
SUDS007	M	1	European	16.27	45.9	n.s.	n.s.	Sleeping	Negative	No	No	Arrhythmia
SUDS008	M	27	European	30.64	460	31%	n.s.	At rest	Hypotensive drugs	Atrial fibrillation, epilepsy, hypertension	No	Arrhythmia with structural changes of the heart
SUDS013	M	63	Indian	23.42	680	120%	Dorsal pain, anorexia	Sleeping	Negative	Renal insufficiency, ICD	Unknown	Sudden cardiac arrest
SUDS015	M	35	European	27.94	470	Normal	Dyspnea	At rest	Alcohol, cannabis	Asthma, migraine	Unknown	Acute heart failure
SUDS019	M	43	European	23.45	370	Normal	Dyspnea	Sleeping	Alcohol (0.32‰)	No	SCD of father aged 68	Acute heart failure
SUDS021	M	31	European	32.08	460	31%	n.s.	Playing football	Negative	No	SCD of grandfather and aunt	Arrhythmia
SUDS023	M	26	African	28.37	460	33%	n.s.	Sleeping	Alcohol (1.63‰)	No	Unknown	Arrhythmia
SUDS024	M	24	European	28.95	320	Normal	n.s.	Physical exercise (outdoor training)	Negative	Unknown	Unknown	Acute heart failure
SUDS026	M	40	European	23.63	540	55%	n.s.	At rest after jogging	Negative	Loss of consciousness	No	Acute heart failure
SUDS027	M	34	European	31.51	430	22%	Dyspnea	Sleeping	Negative	Hypertension	No	Acute heart failure
SUDS028	M	38	European	30.24	470	28%	Dyspnea	Working (builder)	Negative	No	Unknown	Acute heart failure
SUDS030	F	34	European	18.73	320	Normal	Stomach pain, vomiting	At rest	Antihyperacidity drugs	No	Unknown	Acute heart failure
SUDS032	F	21	European	27.34	200	Normal	n.s.	Sleeping	Antipsychotics	Mental retardation	No	SUDEP
SUDS033	M	35	European	28.41	460	39%	n.s.	At rest	Alcohol (1.3‰), amphetamine, hypotensive drugs	Hypertension	Unknown	Acute heart failure
SUDS034	M	47	European	27.76	500	51%	Strong head pain	At rest	Negative	No	No	Acute heart failure
SUDS037	M	20	European	17.51	260	Normal	n.s.	Sleeping	Cannabis, amphetamine	Asthma, epilepsy, spherocytosis	Unknown	Arrhythmia, SUDEP
SUDS038	M	33	European	22.16	410	21%	n.s.	Sleeping	Benzodiazepine, cannabis, cocaine	Unknown	Unknown	Cocaine-induced arrhythmia
SUDS039	M	33	European	35.49	770	127%	n.s.	Working (forester)	Negative	Unknown	Unknown	Acute heart failure
SUDS040	F	28	European	28.03	340	21%	Stabbing back pain, vomiting	At rest	Negative	Atrial fibrillation, ICD	SCD of mother	Arrhythmia
SUDS042	M	51	European	34.16	430	38%	n.s.	Working /physical exercise	Negative	Unknown	Unknown	Acute heart failure
SUDS045	F	50	European	23.46	300	Normal	n.s.	Walking	Negative	No	No	Acute heart failure
SUDS047	F	33	European	22.91	340	Normal	n.s.	At rest	Negative	Unknown	Unknown	Arrhythmia
SUDS049	M	22	European	23.51	300	Normal	n.s.	Sleeping	Negative	No	SD of mother at age 36	Arrhythmia
SUDS050	M	32	African	24.86	380	Normal	Seizures	At rest	Negative	No	Unknown	SUDEP
SUDS051	F	5	African	12.43	92	n.s.	None	Playing	Negative	Slight cognitive developmental disorder	No	Arrhythmia
SUDS056	M	24	European	22.55	430	22%	n.s.	At rest	Negative	Wolff-Parkinson-White syndrome	No	Arrhythmia
SUDS057	M	36	European	26.29	410	Normal	n.s.	Sleeping	Alcohol (0.24‰), antiepileptic drugs	Epilepsy	Unknown	SUDEP
SUDS058	F	11	European	16.28	190	n.s.	None	Swimming	Negative	No	No	Arrhythmia
SUDS060	M	57	European	n.s	549	52%	None	Hiking	Negative	No	Son diagnosed with LQTS	Arrhythmia
SUDS062	M	29	European	27.77	450	31%	n.s.	Working (forester)	Negative	Unknown	Unknown	Arrhythmia

Abbreviations:
F, female; ICD, implantable cardioverter defibrillator; LQTS, long QT syndrome; M, male; n.s., not specified; SCD, sudden cardiac death; SD, sudden death; SUDEP, sudden unexplained death in epilepsy

Table 2 Variants with likely disease-causing effects in the SUD cohort.

Table 2 Variants with likely disease-causing effects in the SUD cohort.

Case	rs-Nr.	Gene	HGVS genomic RefSeq-Nr.	HGVS RefSeq-Nr.	Coding effect	cDNA	Protein change	dbSNP ALLMAF	1000 Genomes EURMAF	ExAC EURNFMAF	ESP EAMAF	Grantham distance score	AGVGD	SIFT	MAPP	MutationTaster	Polyphen2	Disease association
SUDS001	rs139817477	MYLK	NG_029111.1	NM_053025.3	Missense	c.3749G>A	p.(Arg1250His)	0.000000	0.000000	0.000000	0.000581	29	C25	Deleterious	Bad	Disease-causing	Probably damaging	Connective tissue disease
SUDS003	rs199979628	SEMA3A	NG_011489.1	NM_006080.2	Missense	c.196C>T	p.(Arg66Trp)	0.000000	0.001000	0.000810	0.000465	101	C0	Deleterious	Bad	Disease-causing	Probably damaging	BrS
SUDS006	.	RYR2	NG_008799.2	NM_001035.2	Missense	c.12490C>G	p.(Gln4164Glu)	0.000000	NA	NA	NA	29	C25	Deleterious	Bad	Disease-causing	Probably damaging	CVPT
	rs202147766	ACAD9	NG_017064.	NM_014049.4	Missense	c.1258C>T	p.(Arg420Cys)	0.000000	0.001000	0.000000	0.000116	180	C0	Deleterious	Bad	Disease-causing	Probably damaging	ACAD9-deficiency
SUDS008	rs200525962	RYR2	NG_008799.2	NM_001035.2	Missense	c.3380A>G	p.(Glu1127Gly)	0.000600	0.000000	0.000600	0.000832	98	C65	Deleterious	Bad	Disease-causing	Probably damaging	CVPT
SUDS024	rs377763312	BMPR2	NG_009363.1	NM_001204.6	Missense	c.2591C>T	p.(Pro864Leu)	0.000000	0.000230	0.000060	0.000116	98	C65	Deleterious	Bad	Disease-causing	Probably damaging	PAH
SUDS047	rs143662598	EFEMP2	NG_012304.2	NM_016938.4	Missense	c.554G>A	p.(Arg185His)	0.000000	0.000000	0.000030	0.000116	29	C25	Deleterious	Bad	Disease-causing	Possibly damaging	Connective tissue disease
SUDS049	rs34600572	FBN2	NG_008750.1	NM_001999.3	Missense	c.7296G>T	p.(Gln2432His)	0.006990	0.000000	0.000170	0.000233	24	C15	Deleterious	Bad	Disease-causing	Probably damaging	Connective tissue disease
SUDS050	rs139963188	AKAP9	NG_011623.1	NM_005751.4	Missense	c.6134A>G	p.(Asn2045Ser)	0.003790	0.000000	0.000030	0.000000	46	C45	Deleterious	Bad	Polymorphism	Probably damaging	LQTS
SUDS051	.	KCNE5	NG_013241.1	NM_012282.3	Missense	c.184T>A	p.(Tyr62Asn)	NA	NA	NA	NA	143	C65	Deleterious	NA	Disease-causing	Probably damaging	Afib, BrS
SUDS058	.	RYR2	NG_008799.2	NM_001035.2	Missense	c.11441C>T	p.(Ala3814Val)	NA	NA	NA	NA	64	C0	Deleterious	Bad	Disease-causing	Probably damaging	CVPT

Abbreviations:

Afib, atrial fibrillation; AGVGD, align Grantham variation and Grantham deviation; ALLMAF, MAF in all populations based on NCBI dbSNP; BrS, Brugada syndrome; CVPT, catecholaminergic polymorphic ventricular tachycardia; ESP EAMAF, MAF in European-American population in NHLBI GO Exome Sequencing Project; ExAC EURNFMAF, MAF in European (non-Finnish) population Exome Aggregation Consortium; HCM, hypertrophic cardiomyopathy; IVF, idiopathic ventricular fibrillation; LQTS, long QT syndrome; MAF, minor allele frequency; MAPP, multivariate analysis of protein polymorphism prediction; NA, not available; PAH, pulmonary arterial hypertension; SIFT, sorting intolerant from tolerant prediction.

Supplementary information

Neubauer, *et al.* Exome analysis in 34 sudden unexplained death (SUD) victims mainly identified variants in channelopathy-associated genes

Supp. Data Table S1 VUS4-VUS0 in our SUD cohort.

Supp. Data Table S4 VUS4-VUS0 in our SIDS cohort

Case	Gender	Age (years)	dbSNP rs-Nr.	Chromosome	Gene	HGVS Refseq-Nr.	HGVS protein	Coding effect	Location	c.DNA	Protein change	dbSNP ALLMAF	1000 Genomes EURMAF	ExAC EURMAF (non-finnish)	espEAMAF	Grantham distance	AGVGD	SIFT	MAPP	MutationTaster	polyphen2	Coverage	Alternative allele	Subclass based on scoring scheme
SUDS001	M	38	rs139817477	3	MYLK	NM_053025.3	NP_444253.3	missense	exon	c.3749C>T	p.Arg1250His	0.00000	0.00000	0.00000	0.00058	29	C25	deleterious	bad	disease-causing	probably damaging	68	0.33	VUS3
SUDS001	M	38	rs151178348	17	JUP	NM_021991.2	NP_068831.1	missense	exon	c.2207G>A	p.Pro736Leu	0.00000	0.00040	0.00043	0.00023	98	C0	tolerated	good	disease-causing	benign	30	0.47	VUS1
SUDS003	M	17	rs199979628	7	SEMA3A	NM_006080.2	NP_006071.1	missense	exon	c.1966G>A	p.Arg656Trp	0.00000	0.00100	0.00081	0.00047	101	C0	deleterious	bad	disease-causing	probably damaging	69	0.49	VUS3
SUDS005	M	24	rs28763971	6	DSP	NM_004415.2	NP_004406.2	missense	exon	c.8605A>G	p.Ile2869Val	0.00459	0.00000	0.00000	0.00000	29	C0	deleterious	bad	polymorphism	benign	99	0.34	VUS1
SUDS006	F	7	no rs-nr.	1	RYR2	NM_001035.2	NP_001026.2	missense	exon	c.12490C>G	p.Gln4164Glu	0.00000	0.00100	0.00000	0.00012	180	C0	deleterious	bad	disease-causing	probably damaging	54	0.41	VUS3
SUDS006	F	7	rs202147766	3	ACAD9	NM_014049.4	NP_054768.2	missense	exon	c.1258C>T	p.Arg420Cys	0.00000	0.00100	0.00000	0.00012	180	C0	deleterious	bad	disease-causing	probably damaging	25	0.44	VUS3
SUDS006	F	7	rs1013797	14	SYNE2	NM_182914.2	NP_878918.2	missense	exon	c.11944A>G	p.Asn3982Asp	0.00000	NA	0.00148	0.05872	23	C0	tolerated	good	polymorphism	benign	41	0.49	VUS1
SUDS007	M	1	rs139547471	7	ELN	NM_001278939.1	NP_001265868.1	missense	exon	c.2246G>A	p.Gly749Glu	0.00000	0.00010	0.00020	0.00023	98	C0	deleterious	NA	disease-causing	probably damaging	38	0.45	VUS2
SUDS007	M	1	rs35351345	1	CLCNKB	NM_000085.4	NP_000076.2		splice site	c.1297+5G>T	p.?	0.00359	0.00290	0.00899	0.00953	NA	NA	NA	NA	NA	NA	59	0.42	VUS1
SUDS008	M	27	rs200525962	1	RYR2	NM_001035.2	NP_001026.2	missense	exon	c.3380A>G	p.Glu1127Gly	0.00060	0.00000	0.00060	0.00083	98	C65	deleterious	bad	disease-causing	probably damaging	73	0.45	VUS4
SUDS008	M	27	rs137854611	3	SCN5A	NM_001099404.1	NP_001092874.1	missense	exon	c.2204G>T	p.Ala735Glu	0.00000	0.00000	0.00000	0.00000	107	C0	deleterious	bad	disease-causing	benign	61	0.46	VUS2
SUDS008	M	27	rs116888866	6	DSP	NM_004415.2	NP_004406.2	missense	exon	c.7916G>A	p.Arg2639Gln	0.00359	0.00000	0.00008	NA	43	C0	deleterious	bad	disease-causing	probably damaging	38	0.46	VUS1
SUDS019	M	43	rs142877365	10	MYPN	NM_001256267.1	NP_001243196.1	missense	exon	c.3833G>A	p.Arg1278Gln	0.00000	0.00000	0.00069	0.00058	43	C0	deleterious	NA	disease-causing	probably damaging	33	0.39	VUS2
SUDS019	M	43	rs35351345	1	CLCNKB	NM_000085.4	NP_000076.2		splice site	c.1297+5G>T	p.?	0.00600	0.01100	0.00151	0.00953	NA	NA	NA	NA	NA	NA	87	0.47	VUS1
SUDS024	M	24	rs377763312	2	BMPR2	NM_001204.6	NP_001195.2	missense	exon	c.2591C>T	p.Pro864Leu	0.00000	0.00023	0.00006	0.00012	98	C65	deleterious	bad	disease-causing	possibly damaging	41	0.41	VUS4
SUDS024	M	24	no rs-nr.	1	NEXN	NM_144573.3	NP_653174.3	missense	exon	c.1644T>G	p.Asp548Glu	0.00000	NA	NA	NA	45	C0	tolerated	bad	disease-causing	NA	25	0.28	VUS1
SUDS028	M	38	no rs-nr.	6	SYNE1	NM_182961.3	NP_892063	missense	exon	c.19501T>C	p.Asp6502Gly	0.00000	NA	NA	NA	94	C0	deleterious	NA	disease-causing	probably damaging	38	0.49	VUS2
SUDS032	F	21	no rs-nr.	2	BMPR2	NM_001204.6	NP_001195.2	missense	exon	c.409A>C	p.Thr137Pro	0.00000	NA	NA	NA	38	C0	deleterious	bad	disease-causing	benign	161	0.5	VUS2
SUDS032	F	21	rs61757671	7	AKAP9	NM_005751.4	NP_005742.4	missense	exon	c.6037G>A	p.Glu2013Lys	0.00040	0.00000	0.00197	0.00372	56	C55	deleterious	bad	disease-causing	probably damaging	49	0.31	VUS2
SUDS034	M	47	rs77357563	12	TBX5	NM_000192.3	NP_000183.2	missense	exon	c.331C>A	p.Asp111Tyr	0.00220	0.00720	0.00495	0.00488	160	C65	Deleterious	bad	disease-causing	probably damaging	102	0.44	VUS1
SUDS037	M	20	rs78771765	19	DMPK	NM_001288764.1	NP_001275693.1	missense	exon	c.1555G>A	p.Ala519Cys	0.00060	0.00000	0.00195	0.00141	180	C0	deleterious	NA	disease-causing	probably damaging	54	0.43	VUS2
SUDS037	M	20	rs145571020	11	ILK	NM_001014794.2	NP_001014794.1	missense	exon	c.707A>G	p.Asn236Ser	0.00060	0.00000	0.00008	0.00012	46	C45	deleterious	NA	disease-causing	benign	83	0.49	VUS2
SUDS045	F	50	rs28730772	14	MYH6	NM_002471.3	NP_002462.2		splice site	c.2928+5C>T	p.?	0.00020	0.00000	0.00285	0.00267	NA	NA	NA	NA	NA	NA	44	0.032	VUS2
SUDS047	F	33	rs143662598	11	EFEMP2	NM_016938.4	NP_058634.4	missense	exon	c.554C>T	p.Arg185His	0.00000	0.00000	0.00003	0.00012	29	C25	deleterious	bad	disease-causing	possibly damaging	26	0.38	VUS3
SUDS047	F	33	rs142354133	10	ANKRD1	NM_014391.2	NP_055206.2	missense	exon	c.347G>A	p.Thr116Met	0.00080	0.00140	0.00021	0.00012	81	C0	deleterious	good	disease-causing	possibly damaging	32	0.16	VUS1
SUDS049	M	22	rs34600572	5	FBN2	NM_001999.3	NP_001990.2	missense	exon	c.7296C>A	p.Gln2432His	0.00699	0.00000	0.00017	0.00023	24	C15	deleterious	bad	disease-causing	probably damaging	59	0.42	VUS3
SUDS049	M	22	no rs-nr.	11	DCHS1	NM_003737.3	NP_003728.1	missense	exon	c.9115C>G	p.Ala3039Pro	0.00000	NA	NA	NA	27	C0	deleterious	NA	disease-causing	probably damaging	61	0.39	VUS2
SUDS050	M	32	rs139963188	7	AKAP9	NM_005751.4	NP_005742.4	missense	exon	c.6134A>G	p.Asn2045Ser	0.00379	0.00000	0.00003	0.00000	46	C45	deleterious	bad	polymorphism	probably damaging	25	0.36	VUS3
SUDS050	M	32	rs138631004	11	DCHS1	NM_003737.3	NP_003728.1	missense	exon	c.2953G>A	p.Arg985Trp	0.00539	0.00290	0.00035	0.00023	101	C65	deleterious	NA	disease-causing	probably damaging	75	0.44	VUS2
SUDS050	M	32	rs113112855	15	HCN4	NM_005477.2	NP_005468.1		splice site	c.1978+3C>A	p.?	0.00060	0.00000	0.00000	0.00000	NA	NA	NA	NA	NA	NA	50	0.44	VUS2
SUDS051	F	5	no rs-nr.	X	KCNE5	NM_012282.3	NP_036414.1	missense	exon	c.184A>T	p.Tyr62Asn	0.00000	NA	NA	NA	143	C65	deleterious	NA	disease-causing	probably damaging	144	0.43	VUS4
SUDS051	F	5	rs539219309	12	ACAD5	NM_000017.3	NP_000008.1	missense	exon	c.814C>T	p.Arg272Cys	0.00020	0.00000	0.00005	NA	180	C65	deleterious	NA	disease-causing	probably damaging	77	0.49	VUS2
SUDS051	F	5	no rs-nr.	18	GATA6	NM_005257.5	NP_005248.2	missense	exon	c.1648G>A	p.Gly550Arg	0.00000	NA	NA	NA	125	C0	tolerated	NA	disease-causing	probably damaging	50	0.46	VUS2
SUDS051	F	5	rs150710061	1	ACADM	NM_001286043.1	NP_001272972.1	missense	exon	c.1190T>C	p.Ile397Thr	0.00000	0.00047	0.00048	0.00058	89	C0	tolerated	NA	disease-causing	benign	86	0.48	VUS1
SUDS051	F	5	no rs-nr.	4	HADH	NM_001184705.2	NP_001171634.2	missense	exon	c.719C>T	p.Thr240Met	0.00419	0.00000	0.00059	0.00050	81	C0	deleterious	unknown	polymorphism	NA	142	0.34	VUS1
SUDS058	F	11	no rs-nr.	1	RYR2	NM_001035.2	NP_001026.2	missense	exon	c.11441C>T	p.Ala3814Val	0.00000	NA	NA	NA	64	C0	deleterious	bad	disease-causing	probably damaging	90	0.33	VUS3
SUDS062	M	29	rs111748421	13	SMAD9	NM_001127217.2	NP_001120689.1	missense	exon	c.65A>G	p.Leu22Pro	0.00100	0.00000	0.00228	0.00128	98	C65	deleterious	bad	disease-causing	probably damaging	75	0.45	VUS2
SUDS062	M	29	rs142028375	7	SEMA3A	NM_006080.2	NP_006071.1	missense	exon	c.478A>T	p.Ser160Thr	0.00000	0.00000	0.00005	0.00023	58	C0	deleterious	bad	disease-causing	benign	72	0.42	VUS2
SUDS062	M	29	rs35766612	3	TGFBR2	NM_001024847.2	NP_001020018.1	missense	exon	c.1234G>A	p.Val412Met	0.00020	0.00000	0.00187	0.00302	21	C15	deleterious	bad	disease-causing	probably damaging	98	0.45	VUS1

Abbreviations:
AGVGD, align Grantham variation and Grantham deviation; ESPEAMAF, MAF in European American population in NHLBI GO Exome Sequencing Project; ExAC EURNFMAF, MAF in European (non-Finnish) population in exome aggregation consortium; F, female; M, male; MAF, minor allele frequency; MAPP, multivariate analysis of protein polymorphism prediction; Polyphen2, polymorphism phenotyping v2; MutationTaster; 1000 Genomes Project EURMAF, MAF in European population in 1000 Genomes Project; NA, not available; SIFT, sorting intolerant from tolerant prediction

2.4 Manuscript IV

Functional implications of a rare variant in the sodium channel β1B subunit (*SCN1B*) in a five-month old male sudden infant death syndrome (SIDS) case

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Keywords: sudden infant death syndrome, whole-exome sequencing, cardiac channelopathy, *SCN1B*, electrophysiological analysis, whole-cell patch clamp

Personal contribution: Mutagenesis, HEK293 cell culture transfection; whole-cell patch clamp recording, analysis of results, writing the manuscript

Manuscript in preparation

Key teaching points

- Sudden infant death syndrome (SIDS) is defined as the sudden death of a healthy infant younger than one year of age without any obvious cause of death. Despite intensive genetic investigations, underlying pathophysiological mechanism still remains elusive in most of the cases.
- Whole-exome sequencing in a five-month old male infant identified a heterozygous missense variant in the β1B-subunit of *SCN1B*. Electrophysiological recordings of $Na_v1.5$ co-expressed with the two different *SCN1B* isoforms and the variant demonstrated that the variant induces a loss-of-function of $Na_v1.5$ channels.
- The loss-of-function might have contributed to the sudden death event in this infant, however further investigations are needed. This study demonstrates the importance of careful evaluation of likely pathogenic variant identified within next generation sequencing approaches for an accurate interpretation of genetic results.

Introduction

Sudden infant death syndrome (SIDS) is defined as the sudden and unexpected death of an apparently healthy infant younger than one year of age[1]. The occurrence of SIDS is described by a triple risk model involving a critical developmental period in combination with environmental and genetic risk factors, however the pathophysiological mechanisms responsible for SIDS still remain poorly understood[2]. Technical advances in high-throughput massive parallel sequencing recently enabled broad genetic analyses in large SIDS cohorts and identified likely pathogenic sequence alterations in cardiovascular-disease associated genes in up to 30% of SIDS victims[3, 4]. The majority of these genes are reported in cardiac channelopathies, which can cause lethal arrhythmias in absence of any structural changes in the heart and might therefore be one of the most plausible and comprehensible underlying causes of death in SIDS cases[5].

Voltage-gated sodium channels are integral membrane proteins primarily found in cardiac muscle cells where they are involved in the generation and propagation of action potentials[6]. The Na+-current (I_{Na}) is determined not only by the pore-forming α-subunit ($Na_v1.5$), but also by regulatory β-subunits (β1-β4). *SCN1B* is expressed into two isoforms, the transmembrane β1-subunit and the soluble β1B-subunit that includes a retained intron encoding a novel C-terminus, stop codon, and polyadenylation site[7]. Mutations in the voltage-gated sodium channel β-subunit encoding gene *SCN1B* have been reported in multiple inherited cardiac diseases, including long QT syndrome (LQTS), Brugada syndrome (BrS), cardiac conduction defect (CCD), atrial fibrillation (AF), sick sinus syndrome (SSS) and SIDS[8, 9]. In addition, *SCN1B* loss-of-function mutations have been

described in Dravet syndrome (DS), a devastating paediatric epileptic encephalopathy with a high mortality during early childhood, mainly due to sudden, unexpected death in epilepsy (SUDEP)[10]. Here, we report the electrophysiological effect of a rare *SCN1B* β1B-subunit variant identified in the whole-exome sequencing data of a five-month old male SIDS infant.

Case report

The five-month old male infant (weight 6540g; height 64cm; European origin) was found dead in his cot early in the morning in a safe sleeping environment with no evidence of an accidental death. He was born full-term following a normal and uncomplicated pregnancy and was the third-born son of a 34-years old healthy woman. The boy was vaccinated against diphtheria, tetanus, pertussis, and poliomyelitis with a last vaccination 13 days prior to his death. During his months of life, no anomalous clinical events were reported except for a remarkable large head, with a head circumference greater than the 97th percentile. According to his parents, the boy slept a lot and they often had difficulties awakening him.

A comprehensive autopsy investigation revealed normal sizes and structures of all organs with no signs of malformation, malignancy or infections. In addition, microbiological and toxicological screening tests were negative. As the cause of death remained unexplained, the case was assigned to SIDS category I based on the San Diego definition[1].

Ethical approval for this study was provided by the local ethics committee (KEK-ZH-Nr. 2013-0086), and the study was conducted in full conformance with Swiss laws and regulations. Family members were not available for co-segregation analyses due to samples anonymity required by the ethical committee.

Whole-exome sequencing and variant analysis has been performed within a large genetic screening study in 161 SIDS infants[4]. The five-month old boy carried a heterozygous missense variant (rs369588692, c.673C>T, p.R225C) in *SCN1B* (Figure 1). The sequence alteration is located in exon 3A, which is unique to the splice isoform β1B. The variant p.225C was found to be very rare in the European (non-Finnish) population (MAF: 0.01%) in the NHLBI GO Exome Sequencing Project (ESP). In silico protein predictions tools categorized the variant as disease-causing (MutationTaster) and deleterious (SIFT). Based on the American College of Medical Genetic Guidelines[11], the sequence alteration was classified as variant of uncertain significance (VUS) due to the absence of functional studies.

The electrophysiological effects of the two WT β-isoforms (β1 and β1B) and the β1B-subunit variant on $Na_v1.5$ sodium current were assessed using the whole-cell patch clamp technique in transfected HEK2932 cells. Figure 2A shows representative current traces in cells expressing $Na_v1.5$ alone and $Na_v1.5$ plus β1B WT, β1 WT or mutant β1B. Co-expression of $Na_v1.5$ with

β1B-subunit WT or β1B p.R225C showed an increased sodium current density compared to the β1-subunit WT and $Na_v1.5$ alone (Figure 2B, Table 1). In addition, the co-expression of $Na_v1.5$ with β1B-subunit WT induced a depolarizing shift of the steady state inactivation curves leading overall to a gain-of-function (Figure 2C, Table 1). Interestingly, compared to β1B-subunit WT, co-expression of $Na_v1.5$ with β1B-subunit p.R225C variant leads to hyperpolarize the steady state inactivation (Table 2D, Table 1). In conclusion, compared to β1B-subunit WT, β1B-subunit p.R225C will induce a loss-of-function of $Na_v1.5$ channels.

Discussion

Here, we described the results of whole-cell patch clamp analysis of a rare heterozygous missense variant in the β1B-subunit of *SCN1B* found in a male SIDS infant. Sequence alterations in different β-subunits (SCN1B-SCN4B) have been associated with different cardiac diseases and have shown functional evidence in approximately 1% of SIDS cases[8].

In this study, we observed a gain-of-function of the WT β1B-subunit compared to the WT β1-subunit or $Na_v1.5$ alone. The effects of β1B-subunit on $Na_v1.5$ channels are controversial as some groups have reported functional differences between the two isoforms, with or without affecting voltage dependence or channel kinetics, while others reported no effects[9,12]. In addition, the β1B-isoform is not a transmembrane protein, but a soluble protein and little is known about its function[7]. The two β1-isoforms are not only expressed in the human heart, but also in the brain, with the highest amount of the β1B-subunit during embryonic development where the soluble β1B-protein functions as a ligand for β-mediated neurite migration and outgrowth[10]. Variants in β1B were furthermore described as a risk factor for human epilepsy[7], which might be another possible terminal pathway in SIDS infants.

The p.R225C variant is located in the retained region of β1B, which is unique to this isoform. As close located variants have been described in LQTS, BrS, lone atrial fibrillation and idiopathic epilepsy[8-10], the here identified variant represents an interesting candidate sequencing alteration in the pathophysiological mechanisms contributing to the sudden death event. Therefore, the loss-of-function of this variant might have contributed to the vulnerability of this infant in combination with other genetic or environmental risk factors. However, due to the controversial literature reports and our own findings, further investigations are needed to clarify the role and function of these two β1-isoforms and the variant.

In the last years, next-generation sequencing approaches had facilitated the genetic investigation of patients with complex genetic patterns or the identification of underlying genetic causes in large study populations in a time- and cost-efficient manner. The generation of such huge amount of data requires very stringent variant evaluation systems in order to separate false-positive

variants from truly disease-causing sequencing alterations[11]. Several studies have demonstrated that many variants previously associated with cardiac diseases have in fact no or only minor functional effects and are therefore less likely associated with a dominant form of the disease, still they could act as risk modifier[13-15]. Therefore, functional studies are required and recommended for an evidence-based classification and interpretation of the pathogenicity of variants in genetic SIDS studies so far[11,16].

Limitations of this study are related to the functional approaches. Electrophysiological recordings were conducted in a conventional heterologous expression system where the environment is different from adult rod shape cardiomyocytes. Therefore, the effects of many proteins known to associate with the sodium channel complex could not be investigated. In addition, only little is known about the function and physiological role of the splice site variant β1B, emphasizing the need to further investigate the function and expression of this isoform in native tissues.

Conclusion

Electrophysiological investigations of the p.R225C variant showed differences compared to the WT suggesting a loss-of-function of sodium current. To what extent this variant has contributed to the sudden death of the five-month old boy needs to be further clarified. This study highlights the importance of a careful evaluation of likely pathogenic variants identified within next generation sequencing data. Even if variants are categorized as disease-causing according to *in silico* protein predictions tools, the performance of additional functional analyses is essential for an accurate interpretation of genetic results.

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Figures

Figure 1 Sanger sequencing confirmation of *SCN1B* c.673C>T, p.R225C.

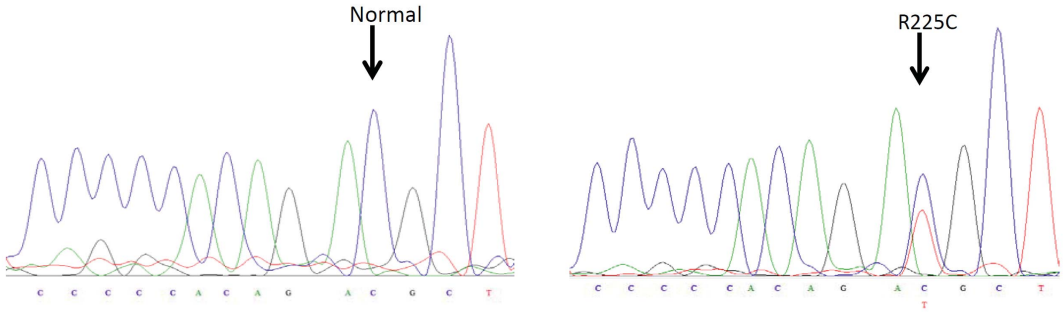

Figure 2 Electrophysiological characteristics of *SCN1B*.

A) Representative traces of sodium current with $Na_v1.5$ alone, β1-subunit WT, β1B-subunit WT, and β1B-subunit p.R225C in HEK293 cells. B) Sodium current density for $Na_v1.5$ alone, β1-subunit WT, β1B-subunit WT, and β1B-subunit p.R225C. C) Voltage dependence of steady-state activation (SSA) for $Na_v1.5$ alone, β1-subunit WT, β1B-subunit WT, and β1B-subunit p.R225C. D) Voltage dependence of steady-state inactivation (SSI) for $Na_v1.5$ alone, β1-subunit WT, β1B-subunit WT, and β1B-subunit p.R225C. In B, C, and D data points represent mean ± SEM. * $p < 0.05$ and ** $p < 0.01$, based on a paired two-tailed Student t-test.

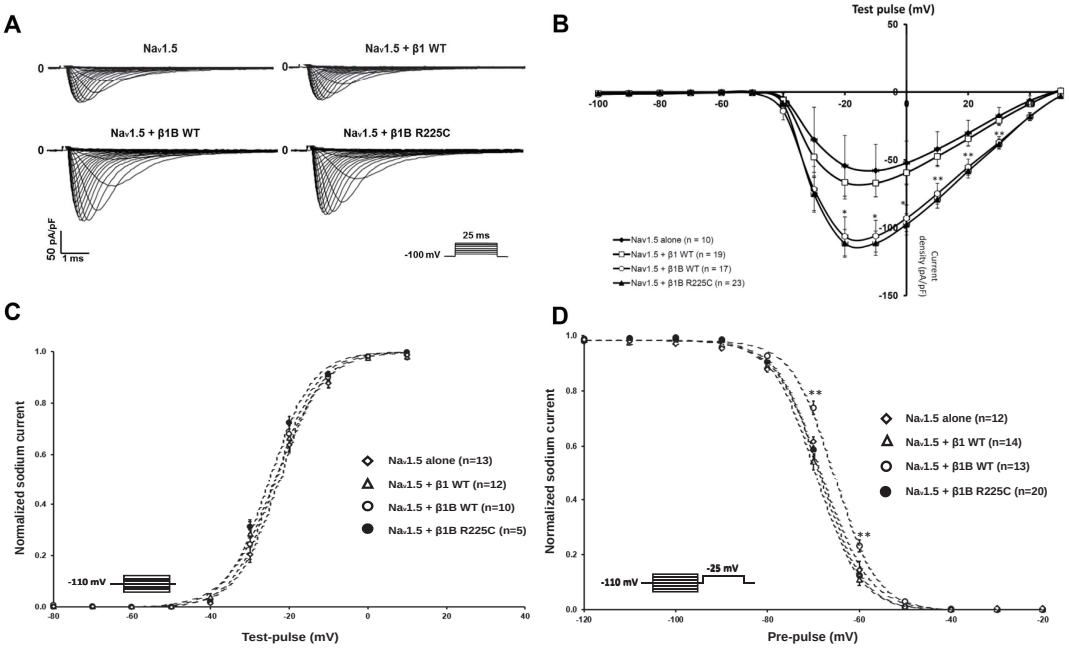

Table 1 Parameters for Na^+-current density, activation and inactivation.

	Current density (pA/pF)		Steady-state activation (SSA)			Steady-state inactivation (SSI)		
	n	(mV)	n	$V_{1/2}$ (mV)	*k*	n	$V_{1/2}$ (mV)	*k*
$Na_v1.5$ alone	10	-57.94 ± 19.34	5	-30.92 ± 0.11	4.03 ± 0.61	10	-68.19 ± 0.59	4.70 ± 0.22
$Na_v1.5$ + β1 WT	19	-67.11 ± 10.04	16	-30.02 ± 1.00	3.32 ± 0.38	19	-68.74 ± 0.48	4.38 ± 0.48
$Na_v1.5$ + β1B WT	17	-106.21 ± 11.71*	17	-30.96 ± 1.13	3.79 ± 0.39	17	-66.12 ± 0.69*	4.64 ± 0.69
$Na_v1.5$ + β1B R225C	23	-111.41 ± 9.04*	21	-30.07 ± 0.85	3.81 ± 0.30	23	-68.24 ± 0.72	4.47 ± 0.72

*$p < 0.05$ *vs*. $Na_v1.5$ alone, compared by paired two-tailed Student *t*-test.

k = slope; n = number of recordings; $V_{1/2}$ = voltage of half-maximal activation/inactivation.

III. DISCUSSION

Sudden and unexplained death in healthy children and young adults is an extremely rare, but very shocking and tragic event for those left behind. The extensive examination and elucidation of these cases is of particular importance in order to identify additional at-risk family members and to prevent similar death cases in the future.

In the current thesis, a main objective was to test whether a post-mortem molecular autopsy procedure provides an additional value in the investigation and detection of lethal genetic diseases which might have initiated or contributed to a sudden death event. As a variety of life-threatening pathophysiological mechanisms might have been involved in the occurrence of a sudden death event, high-throughput massive parallel sequencing represents the most promising tool for additional genetic investigation of these genetically heterogeneous death cases. We continuously improved the DNA library preparation protocol for post-mortem DNA samples that often provide insufficient DNA qualities and quantities for next-generation sequencing. As cardiovascular and metabolic diseases are assumed to be the most promising pathways in life-threatening events, we focused on a list of approximately 200 candidate genes for the data analysis. In our proof-of-principle study, we showed that post-mortem DNA quality is sufficient for next-generation sequencing and that three out of five representative SUD cases carried likely disease-causing sequencing alterations in cardiovascular disease-associated genes[286]. By developing a filtering strategy and a scoring system for the evaluation of variants identified within the exome data of large cohorts, we reported likely pathogenic sequence alterations in 20% of the SIDS and 29.4% of the SUD cases that had remained unexplained so far. Most of the variants were identified in channelopathy-associated genes (SIDS: 45.1%; SUD: 60.1%), followed by cardiomyopathies (SIDS: 35.4%; SUD: 10%), and metabolic diseases (SIDS: 6.45%; SUD: 0%). Most of the identified variants are not functionally characterized so far, and their contribution to the death event is therefore not confirmed. We performed an electrophysiological assay of a candidate variant located in the *SCN1B* gene to examine its effect on the sodium current. The *SCN1B* p.R225C variant induced a loss-of-function of the sodium current and therefore supported the likely pathogenic effect predicted by different *in silico* protein prediction tools. To what extent this variant has actually contributed to the sudden death event of this five-month old male infant needs to further be clarified.

Within the scope of this thesis, a SIDS/SUD biobank was set up at the ZIFM to collect tissue material of the approximately 10 sudden death cases per year which remained without any identifiable cause of death. The ZIFM SIDS/SUD biobank includes tissue samples of 34 SUD

cases and 161 infant cases. The infant cases were collected since 1985 and most of the cases were examined by the same forensic pathologist, ensuring a high level of uniformity in autopsy procedures and case reporting. Therefore, we have a very valuable and large SIDS cohort that can be compared with other international SIDS cohorts. Since 2013, the autopsy procedure of the SIDS and SUD cases at the ZIFM includes the collection of heart (right ventricular outflow tract, left and right ventricular free wall, cardiac septum, left and right atrium, and front left ventricle) and kidney tissues. For each tissue type, one piece is shock frozen in liquid nitrogen and stored in a -80°C freezer, the other one is stored in 70% alcohol.

To our knowledge, we were the first that performed a WES analysis in a large SIDS cohort so far. In the literature, two targeted NGS sequencing approaches in SIDS cases have been described in the meantime. In a proof-of-principle study, Campuzano *et al.* used a targeted NGS approach to investigate the underlying genetic cause in a 11 month-old male SIDS infant[287]. By focusing on 104 genes associated with sudden cardiac death, the study identified seven genetic variations in the genes *AKAP9*, *EN1*, *KCNE3*, *PKP2*, *VCL* and *TTN*. The variant located in *AKAP9* (p.I1643L) showed a rare allele frequency and the position is highly conserved between species, suggesting a potential pathogenic role, especially as other variants in this gene have already been associated with LQTS. Familial segregation analysis showed that the index case's mother and sister carried the same variant, but remained asymptomatic so far. Due to the characteristic incomplete penetrance of LQTS in combination with an increased risk of SCD, β-blocking agents were given in both mutation carriers as a preventive measure of SCD. This study demonstrated for the first time that a molecular autopsy can help to clarify potential pathogenic underlying mechanisms and can identify genetic carriers at risk of SCD. A second study by Hertz *et al.* investigated 47 Danish SIDS cases by using a targeted sequencing approach that covered 100 genes associated with inherited channelopathies and cardiomyopathies[288]. Likely functional effects were identified in 34% of the cases whereas half of the victims carried likely disease-causing variants in genes encoding for ion channels in the cardiomyocytes. Interestingly, two variants in the genes *ANK2* (p.Glu48Gly) and *TRPM4* (p.Trp525*) were also present in our SIDS cohort. These two sequence alterations are interesting candidate variants as these genes have been associated with LQTS, progressive familial heart block and BrS[289]. Compared to the study by Hertz *et al.*, we identified fewer variants with likely functional effects (Hertz *et al.*: 34% vs. our study: 20%). This discrepancy might be explained by the fact, that we used a more stringent variant scoring system in order to include only the most promising variants for further evaluation. Familial co-segregation analysis was neither possible in the study by Hertz *et al.* nor in our study due to sample anonymity required by the ethical committees. Family follow-up would potentially have validated or rejected some of the variants with likely functional effects. The study by Hertz *et al.*

and our study demonstrated that altered ion channel functions causing lethal arrhythmias may represent one of the most plausible and comprehensible cause of death in infants termed as SIDS. Many channelopathies are characterized by incomplete penetrance and variable expressivity, where sudden cardiac death is often the first manifestation of the disease[195]. In contrast, cardiomyopathies are mainly caused by variants in genes encoding desmosomal cell adhesion proteins or in sarcomeric proteins involved in the heart contraction inducing structural heart abnormalities. These structural abnormalities are however often not visible in very young infants, and are therefore another comprehensible cause of death in some of the SIDS infants. Compared to cardiovascular diseases, metabolic diseases seem not to be a major contributor to sudden death events in SIDS cases. Metabolic diseases were detected in 1% of our SIDS cohort, with variants involved in glycogen storage disease and systemic primary carnitine deficiency. As only little functional effect was reported for these variants, they might not have been directly involved in the sudden death event; still they could have contributed as risk factors to the vulnerability of the infants. In conclusion, both NGS studies in large SIDS cohorts demonstrated that undetected cardiovascular diseases can be resolved by molecular autopsy testing in a subset of infants initially diagnosed as SIDS. In general, the etiology of SIDS is very complex and a large proportion of the death cases might rather be caused by a combination of environmental risk factors and genetic predispositions than by a clear genetic disease, making the genetic analysis of these cases almost impossible. Additionally, we have to take into account, that accidental or even intended suffocation is not easy to detect during autopsy investigations, and some of these cases might have been misinterpreted.

In the SUD cohort, consisting of 34 death cases, the detection rate of variants located in cardiovascular disease-associated genes is similar to the findings in other SUD cohorts. In 2014, a study described a first post-mortem WES and gene-specific analysis of 117 sudden death-susceptibility genes for 14 sudden death victims younger than 35 years of age[290]. Eight ultra-rare variants were identified in the genes *CACNA1C*, *JPH2*, *MYH7*, *RYR2*, *VCL* and *TTN* in seven of the 14 cases. Another study reported the identification of likely pathogenic variants in 20% of forensic SUD cases[291]. In the following years, subsequent WES studies in different SUD cohorts described a detection rate between 18 to 44% within cardiovascular disease-associated genes[291-295]. Lethal arrhythmias are considered to be the most plausible cause of sudden death in otherwise healthy individuals, often triggered by different events such as stress, drugs, or physical exertion[296]. Many of these victims were asymptomatic during lifetime and the sudden death was the first manifestation of an undetected cardiac disease. Cardiac channelopathies and cardiomyopathies are characterized by a variable expression and incomplete penetrance, and the initial phenotypic alterations may not be visible at autopsy or may be considered unspecific or

within the normal range[295]. In addition, the diagnosis of many of these cardiac diseases requires ECG measurements which are not possible for sudden death cases, complicating an accurate post-mortem diagnosis of the underlying disease. Therefore, post-mortem molecular autopsy is an efficient strategy to further investigate autopsy-negative sudden unexplained death cases.

In addition to cardiovascular diseases, it has been recognized that a part of the SUD victims suffered from epilepsy which can also lead to life-threatening situations. SUDEP is defined as sudden, unexpected, witnessed or unwitnessed, non-traumatic and non-drowning death in patients with epilepsy with or without evidence for seizure with exclusion of documented status epilepticus and when post-mortem examinations do not reveal any structural or toxicological causes of death[297]. The etiology of SUDEP is suspected to be multifactorial and may be related to autonomic dysfunction, abnormalities in heart rate variability or catecholamine surge and underlying cardiac arrhythmias[298]. Ion channels co-expressed in brain and heart are logical candidates for SUDEP, as defects in intrinsic membrane excitability could underlie both epilepsy and cardiac arrhythmias that precipitated death[299]. The potassium channel *KCNQ1* was the first heart-brain channel identified in SUDEP[300], as mice bearing human disease-linked *KCNQ1* mutations associated with the most common form of cardiac LQTS syndrome exhibit spontaneous, unprovoked seizures that can lead to lethal cardiac arrhythmias[301]. So far, numerous pathogenic variants have been identified in patients suffering from epilepsy and cardiac arrhythmia, mainly in the genes *SCNA5*, *KCNH2* and *RYR2*[302-304]. According to experimental models in mice, the SCN5A protein is expressed in the limbic lobe and may play a relevant role in neuronal activation[305]. Potassium channels, encoded by *KCNH2*, are involved in the regulation of neuronal firing and are particularly active in astrocytes[306]. *RYR2* encoding ryanodine receptors are expressed in the central nervous system and contribute to vesicular transmitter release and postsynaptic spine function[307]. Perturbations of these two channels or of the receptor may confer susceptibility of recurrent seizure activity in combination with cardiac arrhythmias, supporting the emerging concept of a genetically determined cardiocerebral channelopathy which might also be a plausible cause in some of the sudden death events. In our study population, SUDEP was the presumed cause of death in five of the SUD cases, as these victims already had epileptic seizures in their medical history. In two of these cases, likely disease-causing variants were identified in the genes *RYR2* (p.Gln4164Glu) and *AKAP9* (p.Asn2045Ser), concluding that a combination of epileptic seizures and/or cardiac arrhythmia might have contributed to the early death in these two victims.

Advances in next-generation sequencing technologies have reduced the costs of sequencing per base pair about 10-fold, improved accuracy, and greatly increased the speed of generating sequence data. Exome sequencing includes of the capture, sequencing and analysis of all exons

of the human genome which account for approximately 30 million base-pairs within the roughly 3 billion base-pairs of the whole genome[308]. Although only 1-2% of the genome is covered with this approach, mutations within this region are expected to exhibit severe phenotypic consequences. Therefore, WES has rapidly become a common molecular diagnostic screening test for the diagnosis of complex genetic disorders[309]. Finding the causative mutation in a patient can additionally contribute to a more targeted treatment, prevention of further invasive testing, an accurate prognosis of the disorder, and the identification of additional at-risk family members[308]. The main current challenge in exome sequencing studies is still the clinical interpretation of genetic variants identified. The generation of such huge amount of data requires very stringent variant evaluation systems in order to separate false-positive variants from truly disease-causing sequencing alterations. The American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants recommend to classify variants into five categories, ranging from those in which a variant is almost certainly pathogenic for a disorder to those that are almost certainly benign[310]. These categories are based on criteria using typical types of variant evidence, including the evaluation of population data, computational data, functional data, and segregation data. Unfortunately, a lot of these criteria are not applicable for SIDS and SUD, because of the absence of a clear phenotype and family members not available for segregation analyses. Consequently, we had to develop an own scoring system for the evaluation of variants identified in the exomes of sudden death cases, still in accordance with the ACMG guidelines. The majority of variants were categorized as variants of unknown significance (VUS), due to the lack of functional analysis and co-segregation analysis in most of the cases. The requested functional confirmation of all uncharacterized variants in the exome sequencing data is very time-consuming and requires a variety of experiments from protein localizations to mice models. The functional implications of variants located in ion channels or ion channel-associated genes can be investigated by electrophysiological assays. The performed electrophysiological recording in one variant identified within the exome sequencing data of one SIDS victims highlights the importance of a careful evaluation of likely pathogenic variants. Although the variant induced a loss-of-function, the impact on this sudden death event is still not confirmed. In fact it was shown that many variants previously associated with cardiac diseases have no or only minor functional effects[283, 311] and are therefore less likely associated with a dominant form of the disease. Still they could act as risk modifier whereas others might be reclassified as benign based on prospective findings. For example, several mutations in genes previously associated with SIDS were in the meantime identified in exome data from large population studies indicating that many variants might have some pathological influence, but are most likely not the exclusive genetic cause for SIDS[312]. Therefore as a next step, all the variants

identified in NGS-studies need to be verified with functional assays for an evidence-based interpretation of their pathogenicity. Consequently, caution is needed when translating exome sequencing results from research to diagnostic applications.

As molecular autopsies become more widely integrated into criminal prosecution, different issues need to be considered related to ethical, legal, and social challenges. In particular the management of individual results, incidental findings and potential implications of co-segregation analysis in family members need to be regulated. Different to the genetic results of patients, molecular autopsy findings have mainly impact on involved family members and the benefit of the knowledge about a specific inherited disease must be opposed to the emotional burden of learning about inherited diseases. One important challenge concerns the communication of results for variants with unknown significance, as the pathogenicity and exact impact of such variants are very difficult to predict. Therefore, it is recommended that these variants should not be used in family cascade screening as long as their pathogenicity is unknown[313]. In addition, exome sequencing increases the chance of uncovering clinically useful results that are unrelated to the primary aim[314]. Although incidental or secondary findings are not relevant for sudden death victims, they still could have implications on other family members. The general handling of such findings is still under debate as the clinicians and geneticists have to find a balance between medical benefit and the right not to know. According to ACMG, pathogenic mutations found in 56 specific genes, associated with 24 severe conditions, should be reported by the testing laboratory to the clinician, regardless of patient preference[310]. Another issue of genetic testing concerns the situation when testing results are inconsistent with the assumed parentage relationships. For clinical approaches, it has been suggested that information about parentage should not be part of routine genetic test reporting and counseling unless it is specifically requested by the parents[315]. However, how this issue should be handled in sudden death victims, where mainly close relatives benefit from counselling and treatments, is unclear. At last, post-mortem molecular genetic testing is very expensive and as the outcome has no direct benefit for the victims themselves, the insurances do not cover the costs. Therefore, an optimal procedure needs to be established that includes standard post-mortem molecular autopsy investigation in sudden death cases in order to uncover undetected genetic diseases within families and to prevent other death cases in the future.

Post-mortem molecular genetic findings have significant impacts for family-members with the same underlying genetic diseases. Nowadays, minor preventive measures such as life-style modifications, prophylaxis, drug therapy, and implantable defibrillators are available in most cardiac diseases preventing another tragedy within the family[4]. However, the legal and ethical aspects of genetic analysis in forensic post-mortem investigations are complex, as the main

forensic purpose is to determine the cause of death and not its implications for surviving relatives[316]. In contrary to at-risk family members, the detection of potential victims who eventually succumb to SUD poses a significant challenge to the health care system. Although there are no prospectively validated algorithms or "risk factors" to identify these patients before the first arrhythmia episode, there are several well-described disease entities associated with a high risk for SCD[317]. A simple and powerful clinical tool to identify patients at risk is an appropriate family history of sudden deaths before the age of 60 in first-degree relatives. In addition, standard or exercise ECG and echocardiography can identify some patients with arrhythmogenic diseases. Therefore, periodic cardiac examinations are recommended especially for young adults who are participating in competitive sports[318].

3.1 Conclusion

In the current thesis, we were able to demonstrate that post-mortem molecular autopsy investigations represent a valuable approach to identify underlying cardiovascular diseases in approximately one third of all sudden unexplained death cases. In addition, these genetic findings enable the selective investigation of at-risk family members in whom defined genetic counselling and specific treatments could contribute to the prevention of another tragic sudden death. Based on the promising results of this study, our goal is to implement the molecular autopsy investigation into the standard operating procedure for all sudden unexplained death cases at the Zurich Institute of Forensic Medicine. Relatives will only be informed in case of a positive genetic finding in order to avoid unnecessary anxiety in family members. A multidisciplinary approach will be set up, composed of forensic pathologists, cardiologists, and geneticists to provide an optimal offer for relatives including the genetic and cardiac counselling of at-risk family members in accordance with the Swiss recommendations[319].

3.2 Future perspective

Despite tremendous efforts to identify underlying pathogenic diseases in SIDS and SUD cases, 70-80% of the cases still remain elusive after genetic testing, emphasizing the importance of additional research to better understand the pathological mechanisms leading to a sudden death event.

In our study, a negative genetic testing might be due to disease-causing sequence variants within genes not included in our gene list, due to sequence alterations not detectable in WES approaches, such as deep intronic or splice mutations, or due to variants lost during bioinformatics steps. Therefore, one option is to further analyze the huge amount of exome sequencing data in our

SIDS and SUD cases. This approach might identify new genes associated with a particular disease responsible for a life-threatening event within the SIDS and SUD victims. Especially in SIDS cases, the focus on genes involved in the respiratory pathway, epilepsy, or mitochondrial diseases demonstrate interesting candidate pathways[67, 320, 321]. Moreover, copy number variations (CVNs) exceed the read length in NGS applications and are missed by standard alignment algorithms. CVNs are defined as genomic deletions and duplications greater than 1 Kb and are an important cause of genetic variation in the general population contributing to simple Mendelian disorders and complex genetic traits[322]. Although a NGS-based deletion/duplication analysis for up to 46 cardiomyopathy genes in more than 1400 individuals with cardiomyopathies identified clinically significant deletions and duplications in only 0.63% of the individuals[323], the investigation of CNVs might lead to an additional understanding of the pathophysiological mechanisms in sudden death.

Another option is to focus on gene expression which has a significant importance for the understanding of physiological and pathological process and may yield some new insights into mechanisms of sudden death. Up to 75% of the human genome is transcribed into some type of RNA, whereas the protein coding messenger RNA (mRNA) represents only a small proportion[324]. The vast majority of the human genome is transcribed into non-coding RNAs, divided into long RNAs (>200 nt) including ribosomal RNAs and long non-coding RNAs (lncRNA), and small regulatory RNAs (<200 nt) such as microRNAs (miRNA), short interfering RNAs (siRNA), Piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and other short RNAs[325]. Myocardial transcriptome analysis in human cardiovascular diseases has shown that the expression pattern can be clearly distinguished between ARVC, DCM, and healthy ventricular myocardium[326]. Another study demonstrated that myocardial mRNA expression profiles are changed in sarcolemma calcium regulation, apoptosis, and adipogenesis in patients with ARVC compared to DCM patients and controls, suggesting that these molecular pathways may play a critical role in the pathogenesis of ARVC[327]. Furthermore, certain mRNA species, namely those encoding hemoglobin A1/2 and B (*HBA1/2* and *HBB*) as well as pyruvate dehydrogenase kinase 4 (*PDK4*) have been reported to exhibit distinct post-mortem expression patterns in the left ventricular free wall of SCD victims compared to the corresponding tissues from control subjects with non-cardiac causes of death[328]. Moreover, a number of miRNAs have been identified in the healthy adult heart that are highly expressed in non-diseased cardiac tissue and thus likely play a key role in both normal cardiac maintenance and diseases[329, 330]. In contrast to miRNAs, the mechanisms of gene regulation by lncRNAs are much more complex and involve both activation and inhibition of gene expression, as well as modulation of chromatin architecture[331]. In the cardiovascular system, studies have detected lncRNAs that are up- or down-regulated during

acute myocardial infarction and heart failure, whereas others control hypertrophy and cardiomyocyte death by interfering with miRNAs[332-335].

Therefore, the shock-frozen heart tissue samples of our SUD cohort demonstrate a valuable source for transcriptome analysis which might provide a catalogue of genes that show altered expression profiles in sudden death victims or of cardiac disease specific transcription levels of regulatory RNAs.

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APPENDIX

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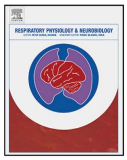

Research paper

Sex-dependent differences in the in vivo respiratory phenotype of the TASK-1 potassium channel knockout mouse

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ABSTRACT

TASK-1 potassium channels have been implicated in central and peripheral chemoreception; however, the precise contribution of TASK-1 for the control of respiration is still under debate. Here, we investigated the respiration of unrestrained adult and neonatal TASK-1 knockout mice (TASK-1$^{-/-}$) using a plethysmographic device. Respiration in adult female TASK-1$^{-/-}$ mice under control (21% O_2), hypoxia and hypercapnia was unaffected. Under acute hypoxia male TASK-1$^{-/-}$ mice exhibited a reduced increase of the respiratory frequency (f_R) compared to wildtypes. However, the tidal volume (V_T) of male TASK-1$^{-/-}$ mice was strongly enhanced. The volatile anesthetic isoflurane induced in male TASK-1$^{-/-}$ and male wild type mice (TASK-1$^{+/+}$) a similar respiratory depression. Neonatal TASK-1$^{-/-}$ mice demonstrated a 30–40% decrease of the minute volume, caused by a reduction of the f_R under control condition (21% O_2). Under hypoxia, neonatal TASK-1$^{-/-}$ mice more frequently stopped breathing (apnea > 3s) suggesting an increased hypoxia-sensitivity. As reported before, this increased hypoxia sensitivity had no influence on the survival rate of neonatal TASK-1$^{-/-}$ mice. In adult and neonatal mice, TASK-1 gene deletion induced a significant prolongation of the relaxation time (R_T), which is a parameter for expiration kinetics. Additionally, screening for mutations in the human TASK-1 gene in 155 cases of sudden infant death syndrome (SIDS) was inconclusive.

In conclusion, these data are suggestive for an increased hypoxia-sensitivity of neonatal TASK-1$^{-/-}$ mice, however, without causing an increase in neonatal lethality. In adult female TASK-1$^{-/-}$ mice respiration was unaffected, whereas adult male TASK-1$^{-/-}$ mice showed a modified breathing pattern. These results are suggestive for sex-specific mechanisms for compensating the inactivation of TASK-1 in mice.

1. Introduction

1.1. Role of background potassium channels in the control of breathing

Precise control of respiration is essential for mammals to maintain adequate blood oxygenation and to eliminate carbon dioxide. Therefore mammals need to sense changes in blood gases (i.e. $O_2/CO_2/H^+$) to ensure the appropriate respiratory response to metabolic challenges (i.e. exercising) or different environments (i.e. high altitude). This ability is called respiratory chemoreception and can be divided into central and peripheral components. Over the last years more and more evidence indicates the importance of background K^+ channels in central and peripheral respiratory chemoreception and in the control of breathing (Bayliss et al., 2015; Buckler, 2015; Kumar et al., 2015). Two-pore-domain potassium channels (K2P channels, KCNK gene family) have been identified as the molecular basis for so-called background K^+ channels (Fink et al., 1996; Goldstein et al., 2001)

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and for some of them (**T**wik-related **a**cid-**s**ensitive **K**$^+$ channels; TASK channels) a key function in central and peripheral chemosensitivity has been proposed (Bayliss et al., 2015; Buckler, 2015). The K2P channels are almost voltage-independent and sensitive to various endogenous neurotransmitters, pharmaceutical compounds (i.e. volatile anesthetics), and physicochemical factors (e.g. CO_2/O_2/pH/redoxpotential) (Goldstein et al., 2001). In excitable cells like neurons this modulation allows K2P channels to dynamically control cell membrane potential and, thereby, electrical excitability. Three TASK subunits (TASK-1; TASK-2 and TASK-3) have been considered to play an important role in pH-dependent homeostatic reflexes like the respiratory response to changes in CO_2/H^+ (Kim et al., 2009; Gestreau et al., 2010).

Inhalation anesthetics are known to activate TASK channels (Talley and Bayliss, 2002) and some of their action is believed to be mediated by TASK channel activation (Lazarenko et al., 2010b). In this respect, all 3 knockout mouse lines (TASK-1$^{-/-}$, TASK-3$^{-/-}$ and TASK-1/3$^{-/-}$) demonstrated reduced halothane- and isoflurane-sensitivity (Linden et al., 2006; Lazarenko et al., 2010b). However, respiration in TASK-1$^{-/-}$ mice in the presence of volatile anesthetics has not been examined until now. Therefore, we investigated the influence of isoflurane on respiration in adult TASK-1$^{-/-}$ mice.

1.2. Impact of TASK channels on central chemoreception

Neurons of the retrotrapezoid nucleus (RTN) located in the ventrolateral medullary surface (VSM) of the brainstem were identified as an essential component of central CO_2 sensitivity (Guyenet, 2014). More precisely, a cluster of Phox2b (Paired-like homeobox 2b)-expressing glutamatergic neurons in the RTN is responsible for this chemosensory function (Dubreuil et al., 2008). Approximately half of these RTN neurons express TASK-2 channels (Gestreau et al., 2010; Wang et al., 2013). The physiological respiratory response to hypercapnia and hypoxia is disturbed in TASK-2 knockout mice but not totally abolished (Gestreau et al., 2010). Beside TASK-2 channels, the proton-sensing G protein-coupled receptor 4 (GPR4) was shown to be an important component for respiratory control by CO_2 (Kumar et al., 2015). In this context, mice with a double knockout for GPR4 and TASK-2 showed a nearly completely abolished CO_2 response demonstrating the importance of these two proteins for central CO_2 detection (Kumar et al., 2015). In contrast to the restricted expression of TASK-2 in a few groups of neurons in the mouse brainstem, TASK-1 and TASK-3 expression was found in many types of neurons, including a variety of putative respiratory neurons in the brainstem: respiratory motoneurons of the brainstem and spinal cord (Karschin et al., 2001), cells of the respiratory rhythm-generating pre-Bötzinger nucleus (pre-BötC), cells of the rostral ventral respiratory group (rVRG) (Washburn et al., 2002), respiratory related hypoglossal motoneurones and the putatively chemoreceptive neurons of the locus coeruleus and serotonergic neurons of the raphe nuclei (Sirois et al., 2000). Although expression of TASK-1 and TASK-3 in these nuclei is well documented, their in vivo relevance of these channels is still not fully understood (Bayliss et al., 2015). In neonatal serotonergic raphe neurons studied in vitro, the TASK-like pH-sensitive K^+ current was virtually absent in either TASK-1$^{-/-}$ or TASK-3$^{-/-}$ or in the TASK-1/3$^{-/-}$ double knockout mice (Lazarenko et al., 2010b). These in vitro findings suggested that TASK-1 and TASK-3 play a role in central chemoreception. However, in vivo all three adult TASK knockout mouse lines (TASK-1$^{-/-}$; TASK-3$^{-/-}$ and double mutant TASK-1/3$^{-/-}$) showed a preserved respiratory response to hyperoxic-hypercapnic (3 −10% CO_2) stimulation. Therefore, it was suggested that neither TASK-1 nor TASK-3 is essential for central respiratory chemoreception in adult mice (Mulkey et al., 2007).

1.3. Functional properties of TASK channels in carotid bodies

The main peripheral chemoreceptors are the carotid bodies located in the vicinity of the two carotid arteries and another cell population on the underside of the aortic arch (Buckler, 2015). The carotid body is composed of sensory cells (type I), which respond to hypoxia or acidosis by depolarizing the cell membrane and thereby activating voltage dependent Ca^{2+} entry. The substantive rise of the intracellular Ca^{2+} $[Ca^{2+}]_i$ in the type I cells induces neurosecretion (mainly adenosine-tri-phosphate (ATP)) and excitation of the adjacent afferent nerve via ligand-gated ATP-receptors (P_2X_2/P_2X_3) (Rong et al., 2003). The type I cells express many different ion channels including TASK channels, an uncharacterized Na^+ conductance, Cl^- channels, L-and N-type Ca^{2+} channels, voltage-gated K^+ channels and a large conductance calcium activated K^+ channel (maxi-K channels) (Buckler, 2015). Despite intensive research, the functional contribution of all these channels in chemoreception is not well understood. At resting state, the potassium conductance of type I cells is mainly driven by TASK-1 and TASK-3 channels. Closure of these TASK channels upon exposure to acidosis or hypoxia induces membrane depolarization as an initial signal of the signaling cascade underlying chemo-sensation (Buckler et al., 2000; Buckler, 2015). In contrast to Mulkey and coworkers (Mulkey et al., 2007), another study suggested slightly impaired respiratory response to hypoxia and hypercapnia in TASK-1$^{-/-}$ and TASK-1/3$^{-/-}$ mice (Trapp et al., 2008).

1.4. Sex-dependent differences in respiration and TASK channel expression

In recent years considerable evidence has accumulated showing that sex-hormones can influence respiratory function in animals and humans (Behan and Wenninger, 2008). In this respect, female mice displayed increased ventilation compared to male mice, which is thought to be most likely female sex-hormone dependent (Schulz et al., 2002; Soliz et al., 2009). For instance, erythropoietin led to an increased ventilatory response to hypoxia in female mice and women compared to male mice respectively men (Soliz et al., 2009). In addition, adult female mice often show improved adaptation to different gene deletions. For example, only male mice with the serotonin transporter knockout showed an impaired CO_2-stimulated respiratory response (Li and Nattie, 2008). Sex-dependent differences related to TASK channel function has also been demonstrated. Male TASK-1$^{-/-}$ mice exhibited an enhanced acoustic response (Linden et al., 2006) and impaired motor control (Linden et al., 2008) in comparison with their wild type counterparts whereas female TASK-1$^{-/-}$ mice responded virtually normal. Besides sex hormone specific effects, age-dependent changes of the expression of TASK channels has been shown in various organs (Kanjhan et al., 2004; Kim et al., 2012a).

Since the precise role of TASK-1 for the control of respiration is still a matter of debate, we investigated respiration in adult TASK-1$^{-/-}$ mice and neonatal TASK-1$^{-/-}$ of both sexes under various respiratory stimuli.

1.5. Impaired chemoreception in sudden infant death syndrome (SIDS)

In the last few decades, there have been major advances in the understanding of SIDS achieved by recognizing its relationship to sleep and homeostasis, environmental and genetic risk factors, and biochemical and molecular abnormalities (Kinney and Thach, 2009). In this regard, impaired functions of proteins involved in central or peripheral chemoreception seem to play a role in SIDS. (Garcia et al., 2013a; Porzionato et al., 2013). In our study neonatal TASK-1 knockout mice exhibited restricted ventilation under both

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control (21% O_2) and hypoxic conditions. We, therefore, performed whole-exome sequencing in a Swiss SIDS cohort to possibly identify rare variants in *KCNK3* (TASK-1 gene).

2. Materials and methods

2.1. TASK-1 potassium channel knockout mouse (*TASK-1*$^{-/-}$)

The TASK-1$^{-/-}$ mice have been generated as described earlier (Aller et al., 2005) and were backcrossed for seven generations into the C57Bl/6J genetic background. The TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice were derived from C57Bl/6J heterozygous breeding pairs. For genotyping, tail biopsies were performed; DNA was extracted and tested for the presence of wildtype and mutant alleles. The used oligonucleotide (Invitrogen, Karlsruhe, Germany) for the wild-type allele gave rise to an amplicon with the length of 300 base pairs sense primer: (5′CGTTGGCTCTCATCGTGTGCACC 3′) on exon1 and antisense primer: (5'CGCGGCGAGTGTAGTTAC 3′) on intron 1. For the TASK-1 KO the sense primer (5′CCTTCTATCGCCTTCTTCACG 3′) on the neomycin cassette and antisense primer (5′TGATGGCGAAGTAGAAGGAGC 3′), on exon 1 created an amplicon of 344 base pairs.

The mice were kept on a 12:12 h light/dark cycle beginning at 8 am, and had free access to chow (standard diet) and water. The experiments were done during the light cycle (from 8 am until 8 pm).

All animal experiments were performed according to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health and were approved by the local councils for animal care according to the German law for animal care.

For the experiments, either female or male mice of the same age (50–120 days) were used. Female TASK-1$^{+/+}$ mice had an average weight of 22.15 ± 0.24 g (n = 10) and the TASK-1$^{-/-}$ mice of 21.31 ± 0.65 g (n = 10; p = 0.42). Male TASK-1$^{+/+}$ mice were significantly heavier at the same age and had an average weight of 28.55 ± 0.39 g (n = 26) and the TASK1$^{-/-}$ mice of 25.57 ± 0.50 (n = 26; p < 0.001). Neonatal mice of both sexes were 3–7d old and had an average body weight of 3.48 ± 0.29 g (TASK-1$^{+/+}$; n = 10; 4 female, 6 male mice) and 3.96 ± 0.09 g (TASK-1$^{-/-}$; n = 8; p = 0.148; 3 female and 5 male mice).

2.2. Whole body plethysmography

The respiratory data were obtained using whole body plethysmography for unrestrained animals (emka technologies, Paris, France). The volume of the plethysmography chamber was 250 ml for adult mice and was reduced by an inlay to a volume of 40 ml for the respiratory measurements of the neonatal mice. The animals were free to move, had free access to water and were not anaesthetized. A constant flow pump connected to the animal chamber ensured continuous flow in the range of 0.5 ± 0.05 l/min of fresh air thereby preventing CO_2 accumulation. For the measurement of the neonatal animals the continuous flow was reduced to 0.25 ± 0.05 l/min. The experiments on adult mice were performed at room temperature (22 ± 1.5° C). During the experiments with neonatal mice, the chambers were put on heating pads to maintain an ambient temperature in the chamber of 28° ± 1° C. Neonatal mice were placed on styroform inlays to reduce heat loss. The calibration of the plethysmograph was performed after the chambers were heated up. The hypoxia or hyperoxic-hypercapnic gas mixture was applied into the animal chamber by the above mentioned flow pump. The O_2 content was controlled by an oximeter (Oxydig, Drägerwerk, Lübeck, Germany).

Prior to the experiment, animals were placed into the plethysmography chambers for a minimum of one hour to allow acclimatization. After one hour of acclimatization, the adult mice became adapted to the experimental setting and the rate of sniffing and movements became usually very low allowing starting the experiment. Sniffing periods, which are mostly accompanied by body movements during the experiment, were excluded from analysis by applying a cut-off for the respiratory frequency of 350 beats/min. The acclimatization time for the neonatal mice was reduced to 10 min to avoid hypothermia.

The respiration patterns of the mice were detected by a pressure transducer coupled to an amplifier (emka technologies, France). The signal was recorded and analyzed with IOX2 software (emka technologies, France). The following respiratory parameters were registered: respiratory frequency (f_R), tidal volume (V_T), minute ventilation (MV), peak expiratory flow (PEF), inspiratory flow (PIF), expiration time (T_E), inspiratory time (T_I) and relaxation time (R_T). Beside the respiratory frequency all measured respiratory parameters (V_T, MV, PEF, PIF) were normalized to the body weight to make them comparable between mice of different body weights. The relaxation time (R_T) represents the time for the expiratory area to decline to 33.3% of the total expiratory area or in other words the time it takes the box pressure signal to drop from its peak to 1/3 of its peak value.

Calibration of the system for every chamber was performed before every experiment according to the manufacturer's protocol: First the gain was adjusted for the appropriate amplification (200–500 for mice) at "0 V" for the low value. Then the gain was adjusted for the high value by injecting 1 ml (for adult mice) air with a syringe into the chamber. For the neonatal experiments, the amplification was 500 and the high value was defined by injecting 0.5 ml air into the chamber.

During the experiments, respiratory parameters were continuously measured and average values were calculated every 1 min. In most analyses, these 1-min mean values were averaged for given periods. All experimental series using adult mice started with a control period with room air (21% O_2) for 30 min. In the hyperoxic-hypercapnic experimental series, the gas was changed to 100% O_2 for 15 min. Then, the gas mixture was changed to 5% CO_2/95% for a period of 15 min. In the hypoxia series a hypoxic gas mixture was created by adding nitrogen to room air. In the stepwise hypoxia series, every 15 min the oxygen concentration was stepwise reduced from 21% to a minimum of 7% and then returned to room air (21% O_2). In the acute hypoxia series, the mice were kept after a control period (21% O_2) of 30 min for 15 min under the hypoxic gas mixture (10% O_2). For the neonatal animals, a different experimental protocol with shorter periods (5 min instead of 15 min) and a higher minimal O_2 concentration (11% O_2) was used, because the neonatal animals were much more sensitive towards hypothermia and hypoxia.

2.3. SIDS study population and sequencing

This study was approved by the local ethics committee of the Canton Zurich, Switzerland (KEK-ZH-No. 2013-0086). Whole-exome sequencing data of a Swiss sudden infant death syndrome (SIDS) cohort (unpublished data) were used to investigate rare variants in *KCNK3* (TASK-1 gene). Sequences were aligned to the reference genome (GRCh37/hg19) using Burrows-Wheeler transform (Li and Durbin, 2010). The SIDS cohort consisted of 155 cases (94 males; median age: 17.4 ± 10.7 weeks) collected between 1985 and 2014 at the Zurich Institute of Forensic Medicine (ZIFM), Zurich, Switzerland. The classification of SIDS cases has been performed according to generally accepted international definitions of SIDS, including a complete autopsy, review of the circumstances of death, and examination of the clinical history (Krous et al., 2004). Genomic

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DNA of the SIDS infants was obtained from tissues stored in alcohol or from alcohol-fixed and paraffin-embedded tissue blocks. In most of the cases kidney or tongue was used (otherwise heart, muscle or brain) because of reported good post-mortem DNA stability in these tissues (Bar et al., 1988). Exome sequencing was performed with the SureSelectXT target enrichment and SureSelectXT All Exon V5+UTR's kits (Agilent Technologies AG, Basel, Switzerland). Sequencing as wells as sequence alignment and variant calling were performed at the Functional Genomics Center Zurich (FGCZ) Switzerland. Sequencing was done on the Illumina HiSeq2500 platform (Illumina Inc., San Diego, USA), generating 2×100 bp paired-end reads. Alamut Visual Version 2.7.1 (Interactive Biosoftware, Rouen, France) was used to manually check exon 1 and 2 of *KCNK*3.

2.4. Statistics

Data are shown as mean values ± SEM from n observations in the figures. Analysis for parametic and nonparametic data were performed by Shapiro-Wilk test. Descriptive statistics with differences between the two genotypes or between male and female sex are given as median and interquartile range (IQR) in the table. The median (V_T, MV,T_i,T_E,PIF,PEF and fR) of the two groups (between TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice or between sexes) were compared by Man-Whitney test. To compare respiratory parameters during control and experimental periods within one group, a repeated measures Analysis of Variance (ANOVA) was used. A $p < 0.05$ was considered to be significant. Statistical analysis was performed using SPSS version 22 (IBM, NY, USA) and Numbers 09 version 2.1 (Apple Inc., Cupertino, CA, USA) software.

In the figures the following symbols were used to indicate statistical significance: "⋆" between TASK-$1^{+/+}$ and TASK-$1^{-/-}$mice (unpaired); "☆" significant differences between control and experimental period (within a given genotype) (paired). In the table, p-values between genotypes are shown directly in the table and the following symbols indicate statistical significance between the sexes within one genotype: "#" p-value < 0.01 between female and male TASK-$1^{+/+}$; "##" p-value < 0.01 between female and male TASK-$1^{-/-}$; "*" p-value < 0.05 between female and male TASK-$1^{+/+}$; "**"p-value < 0.05 between female and male TASK-$1^{-/-}$.

The percentages of experimental break-off between neonatal TASK-$1^{-/-}$ and TASK-$1^{+/+}$ mice during the hypoxia challenge were compared with Kaplan–Meier analyses. Log rank and Chi square test were used to investigate data collected at the level of nominal or ordinal parameters.

3. Results

3.1. Sex-dependent respiratory phenotype of adult TASK-$1^{-/-}$ mice under normoxia

The respiration of unrestrained TASK-$1^{-/-}$ mice was measured using a plethysmographic device. Under control conditions ventilation of wildtype (WT) female mice was higher compared to male mice. In TASK-$1^{-/-}$ animals, however, male animals displayed an enhanced minute volume resulting in the loss of the sex-specific ventilation difference. Under normoxia all respiratory parameters, except for the respiratory frequency (f_R), were significantly different in male TASK-$1^{-/-}$ mice compared with male WT mice (Table 1). By contrast, in female mice the only genotype-related difference in respiration was noted in the relaxing time (R_T) and expiration time (T_E). Interestingly, male (but not female) TASK-$1^{-/-}$ had a lower body-weight than their WT counterparts. Table 1 summarizes the basic respiratory parameters under control conditions (21% O_2).

3.2. Hypoxia-induced respiratory response in TASK-$1^{-/-}$ mice

In Fig. 1 the effect of acute hypoxia (15 min) in male TASK-$1^{-/-}$ and WT mice is shown. Male TASK-$1^{-/-}$ mice did not show a physiological increase of the respiratory frequency under acute hypoxia (Fig. 1, Table 2). However, acute hypoxia induced a strong increase of the tidal volume in male TASK-$1^{-/-}$ mice leading to a normal increase of the minute volume. In contrast to male WT mice male TASK-$1^{-/-}$ mice showed an increased expiration time (T_E) and relaxation time (R_T) under acute hypoxia. In summary, these findings are indicative for a modified, but not severely impaired respiratory answer in male TASK-$1^{-/-}$ mice under acute hypoxia.

To investigate the hypoxia response in more detail, a protocol of stepwise hypoxia was performed in male and female mice. With regard to the main respiratory parameters (minute volume (MV), respiratory frequency (f_R), tidal volume (V_T)), female TASK-$1^{-/-}$ mice were not different from female WT mice (Fig. 2A, Table 3A). As an indicator for a mildly modified breathing pattern, the relaxation time (R_T) of female TASK-$1^{-/-}$ mice was prolonged under control and stepwise hypoxia (Fig. 2B, Table 3A). In male TASK-$1^{-/-}$ mice ventilation was enhanced under control and stepwise hypoxia due to an increased and highly variable tidal volume (Fig. 2A, Table 3B, Fig. A.1 A /B). Additionally, in male TASK-$1^{-/-}$ mice, the depth of breath was strongly increased, as evidenced by a higher PIF, PEF and V_T, whereas the f_R −response seemed to be reduced (Fig. 1, 2B, Table 3B, Fig. A.1A/B).

3.3. Hypercapnia-stimulated response in TASK-$1^{-/-}$ mice is unaffected

Under a hyperoxic-hypercapnic challenge female and male TASK-$1^{-/-}$ mice exhibited an unaffected respiratory response compared with their WT counterparts (Fig. 3). Similar to the hypoxia series (Fig. 1/2A), male TASK-$1^{-/-}$ mice exhibited increased ventilation under hypercapnia, mainly due to an augmented tidal volume (Fig. 3, Table 4). The relative increase of respiration under hypercapnia was similar in males of both genotypes (data not shown). In contrast to acute hypoxia, CO_2 stimulation reduced the genotype-dependent differences in R_T in female TASK-$1^{-/-}$ mice (Fig. 3, Table 4). Taken together, these data suggest a similar breathing pattern and normal respiratory responses to hyperoxic-hypercapnia in TASK-$1^{-/-}$ mice.

3.4. Isoflurane-induced respiratory depression in male TASK-$1^{-/-}$ mice

In the next experimental series the respiratory influence of isoflurane in male TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice was investigated. The global respiratory response of TASK-$1^{-/-}$ mice to isoflurane was similar to wildtype mice, in particular the depression of f_R (Fig. 4A, Table 5A). However, a more detailed analysis revealed slight but specific changes in TASK-$1^{-/-}$ mice: At low concentrations of isoflurane (0.4–0.8%) the genotype-dependent difference in respiration (as observed under control conditions) disappeared. Only at a concentration of 0.8% isoflurane a significantly higher relative respiratory depression was seen in the KO mice (data not shown). At higher concentrations of isoflurane (1-1.5%), it seemed that WT mice displayed a stronger decrease in respiration compared to TASK-$1^{-/-}$ mice. However, the relative decrease of respiration by isoflurane (1–1,5%) was not different between the genotypes (data not shown). Next, we investigated the respiratory CO_2 and hypoxia response under isoflurane. The hypercapnia response was completely abolished at 1% isoflurane in both genotypes (Fig. 4B, Table 5B). Furthermore, also the hypoxia-stimulated ventilation

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Table 1
Respiratory parameters (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV); peak expiratory/inspiratory flow (PEF/PIF); expiration/inspiration time (T_E/T_I); relaxation time (R_T)) of TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice under control condition (21% O_2): Genotype-related differences of both sexes are listed in the table (p-values: differences between genotypes). Data are presented as median and interquartile range (IQR).

	FEMALE			MALE		
	TASK-1$^{+/+}$ (n = 10) Median (IQR)	**TASK-1**$^{-/-}$ (n = 10) Median (IQR)	p-value	**TASK-1**$^{+/+}$ (n = 28) Median (IQR)	**TASK-1**$^{-/-}$ (n = 27) Median (IQR)	p-value
V_T (µl/g)	8.47 (6.87–8.71)#	8.92 (7.51–10.62)	0.257	6.41 (4.96–7.15)	6.91 (6.1–9.18)	0.02
f_R (beats/min)	205.8 (191.9–231.8)	189.6 (173.4–210.9)**	0.112	206.7 (188.3–223.5)	232.8 (198.8–253.1)	0.05
MV (ml/g/min)	1.81 (1.38–1.92)*	1.61 (1.39–2.16)	0.762	1.26 (1.04–1.53)	1.68 (1.37–2.25)	0.005
PEF (ml/g/s)	0.12 (0.097–0.13)#	0.104 (0.092–0.12)	0.45	0.076 (0.065–0.086)	0.115 (0.088–0.159)	<0.001
PIF (ml/g/s)	0.197 (0.148–0.231)#	0.192 (0.162–0.253)	0.65	0.111 (0.082–0.129)	0.218 (0.137–0.266)	<0.001
T_E (ms)	183.3 (176.7–190.4)	210.3 (193.9–240)**	0.041	195.3 (174.9–225.1)	178.9 (145.8–206.7)	0.041
T_I (ms)	86.82 (76.27–99.83)*	83.24 (70.35–95.62)	0.406	101.7 (89.68–112.95)	67.37 (59.48–90.03)	<0.001
R_T (ms)	114.6 (100.4–118.3)	136.2 (122.2–162.2)**	0.01	101 (94.4–107.8)	116.1 (104.1–133.4)	0.008
Body weight (g)	22.18 (21.8–22.8)#	21.43 (19.4–23.25)##	0.427	28.75 (27.05–29.6)	25.6 (23.95–26.65)	<0.001

p-value < 0.01 between female and male TASK-1$^{+/+}$ mice.
p-value < 0.01 between female and male TASK-1$^{-/-}$ mice.
* p-value < 0.05 between female and male TASK-1$^{+/+}$ mice.
** p-value < 0.05 between female and male TASK-1$^{-/-}$ mice.

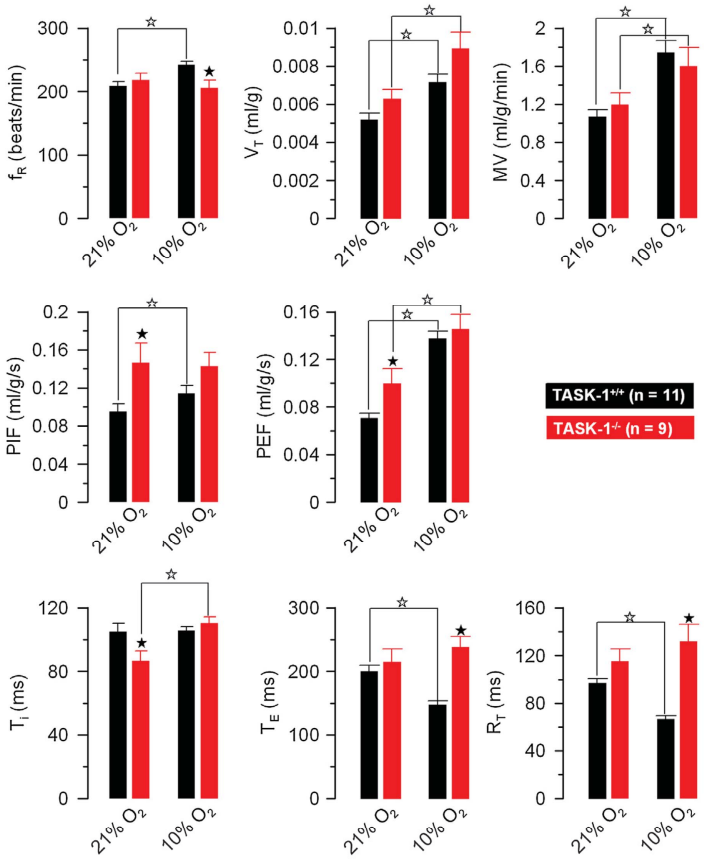

Fig. 1. Respiratory response of male TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice under acute hypoxia (10% O_2/15 min).
In the upper row changes of respiratory frequency (f_R), tidal volume (V_T) and minute volume (MV) are depicted. The middle row shows peak expiratory flow (PEF), peak inspiratory flow (PIF). Higher PIF/PEF under control conditions indicated deeper breath in male TASK-1$^{-/-}$ mice.
In the lower row expiration/inspiration time (T_E/T_I) and relaxation time (R_T) are shown. Data are shown as mean ± SEM. $P < 0.05$: ⋆ significant differences between male TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice; ☆significant differences between control and experimental period (within a given genotype).

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Table 2
Effect of acute hypoxia (10% O_2) on respiratory parameters (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV); peak expiratory/inspiratory flow (PEF/PIF); expiration/inspiration time (T_E/T_I); relaxation time (R_T)) in male TASK-$1^{+/+}$ (n = 11) and TASK-$1^{-/-}$ mice (n = 9). Data are presented as median and interquartile range (IQR).

		21% O_2		10% O_2		
		Median (IQR)	p-value	Median (IQR)	p-value	21% v. 10% O_2
V_T	+/+	5.49 (3.87-6.32)	0.205	6.9 (5.98-8.01)	0.091	<0.001
	-/-	6.36 (6.6-6.94)		8.53 (7.87-9.25)		0.001
f_R (beats/min)	+/+	196 (193.1-232.9)	0.526	239 (231.1-253)	0.003	0.007
	-/-	224 (187.9-242.2)		205 (187.9-209.2)		0.417
MV (ml/g/min)	+/+	1.13 (0.84-1.23)	0.526	1.5 (1.46-2.03)	0.673	<0.001
	-/-	1.23 (0.86-1.41)		1.77 (1.61-2)		0.012
PIF	+/+	0.104 (0.06-0.11)	0.078	0.112 (0.09-0.13)	0.121	0.042
	-/-	0.13 (0.083-0.16)		0.145 (0.11-0.17)		0.777
PEF	+/+	0.07 (0.056-0.08)	0.105	0.13 (0.125-0.15)	0.260	<0.001
	-/-	0.096 (0.07-0.11)		0.15 (0.14-0.17)		0.002
T_I	+/+	106 (92.7-118.07)	0.024	108 (97.8-112.27)	0.573	0.908
	-/-	89 (76.2-102)		109 (102.7-118.3)		0.002
T_E	+/+	204 (174.5-227.6)	0.888	146 (134.7-162.8)	<0.001	0.001
	-/-	196 (188.6-225.3)		228 (198.9-284.7)		0.281
R_T	+/+	97 (85.4-110.6)	0.139	64.2 (55.47-75.8)	<0.001	<0.001
	-/-	107 (103.4-127)		117 (96.5-154.7)		0.29

Genotype-related differences are listed in the table (*p*-values: differences between genotypes).
21% O_2 v. 10% O_2: *p*-values between control and experiment (hypoxia) of either TASK-$1^{+/+}$ or TASK-$1^{-/-}$ mice.

was completely absent at 1% isoflurane in both genotypes (data not shown).

3.5. Impaired ventilation in neonatal TASK-$1^{-/-}$ mice

Since disturbances of the respiratory control are of clinical relevance in neonatal humans, we performed an additional series of experiments to test for a possible respiratory phenotype in neonatal TASK-$1^{-/-}$ mice. In contrast to adult mice, neonatal TASK-$1^{-/-}$ mice of both sexes displayed reduced ventilation (30–40% reduction) under control condition (21%O_2) compared to their WT counterparts (Fig. 5 A/B, Table 6). This reduction in MV was mainly caused by a severely decreased f_R (Fig. 5A/B). Hypoxia suppressed respiration in both genotypes to nearly the same extent. Interestingly, six out of eight neonatal TASK-$1^{-/-}$ mice stopped breathing (apnea period > 3 s) during the hypoxic stimulation or in the post-hypoxic control period indicating increased hypoxia-sensitivity (Fig. 5C). A considerably lower PIF in neonatal TASK-$1^{-/-}$ mice under normoxia and hypoxia indicated a reduced depth of breath (Fig. 5D, Table 6). In neonatal TASK-$1^{-/-}$ mice, the relaxation time (R_T) was prolonged similar to the situation in adult TASK-$1^{-/-}$ mice (Fig. 5D, Fig. 2B). In summary these results suggest that TASK-1 is an important component of respiratory control in neonates under hypoxia.

In the light of these results a search for mutations in the human TASK-1 gene in 155 sudden infant death syndrome (SIDS) cases post-mortem was performed. This analysis didn't reveal mutations in the two exons of human TASK-1 gene in these SIDS cases indicating that TASK-1 mutations play probably no role in SIDS.

4. Discussion

4.1. The sex- and age-dependent respiratory phenotype of TASK-$1^{-/-}$ mice

The key findings of this study were the sex- and age-related respiratory phenotype of TASK-$1^{-/-}$ mice. Neonatal TASK-$1^{-/-}$ mice of both sexes exhibited a 30–40% diminished ventilation. In adult animals, enhanced ventilation was observed in male TASK-$1^{-/-}$ mice. This result is in contrast to another study, where no such increase in respiration was described (Trapp et al., 2008). A possible explanation for this discrepancy could be differences of the genetic background of the mice. Many studies have highlighted variations under control (21% O_2) and in hypoxia- or hypercapnia-stimulated respiration between different mouse or rat strains (Tankersley et al., 1994; Cramer et al., 2015). In this respect a strain-dependent expression of different potassium channels (Kir2.3/TASK-1/Kv1.4) in the nucleus of the solitary tract (NTS) has been demonstrated in rats and was suggested to be responsible for the severely blunted ventilatory CO_2 chemoreflex in brown Norway rats (Martino et al., 2014). Trapp and coworkers did use mice that were "mainly of the C57BL/6 background"; we used mice after at least seven generations of backcross into the C57Bl/6J genetic background.

An additional finding was the significant lower body weight in male TASK-$1^{-/-}$ mice, which was not observed in female TASK-$1^{-/-}$ mice. This result could reflect sex-related differences in fat metabolism (Wang et al., 2006). However, another study found no difference in the body weight of male TASK-$1^{-/-}$ mice (Linden et al., 2006). A temperature-dependent influence of TASK-1 channels on fat metabolism has been demonstrated (Pisani et al., 2016). Therefore, slightly different temperature of animal facilities could be responsible for the discrepancy in the body weight of male TASK-$1^{-/-}$ mice. In addition other factors like a compromised food intake, higher metabolic rate, or/and increased energy loss may contribute to the lower body weight in the male TASK-$1^{-/-}$ mice. However, these factors were not investigated in our study, but further studies addressing these points are planed for the future (e.g. metabolic cage).

What are possible mechanisms underlying the increased ventilation of male TASK-1 knockout mice?

It is known that female mice frequently display increased ventilation compared to male animals (Schulz et al., 2002; Soliz et al., 2009). This physiological sex-dependent difference was not observed in TASK-$1^{-/-}$ mice because of enhanced ventilation in male knockouts. This phenotype could be caused by sex-dependent differences of K^+ channel function as reported for K_{ATP} channel activity in the pre-Bötzinger complex of neonatal mice (Garcia et al., 2013b). It is conceivable that similar to the above mentioned K_{ATP} channels TASK-1 channels also display sex- and age-dependent expression levels in respiration-related central neurons. In agreement with sex-specific expression levels of K^+ channels, TASK-$1^{-/-}$ mice showed a sex- and age-dependent adrenal phenotype (Heitzmann et al., 2008), a sex-specific acoustic response (Linden et al., 2006), and a sex-specific impairment of motor performance (Linden et al., 2008). A direct action of sex hormones on TASK channel expression has been demonstrated in neuroblastoma N2A cells, in which 17ß-estradiol downregulated

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Table 3A
Respiratory parameters (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV); peak expiratory/inspiratory flow (PEF/PIF); relaxation time (R_T)) of female TASK-1$^{+/+}$ (n = 8) and TASK-1$^{-/-}$ mice (n = 8) under stepwise hypoxia: Genotype-related differences of both sexes are listed in the table (*p*-values: differences between genotypes). Data are presented as median and interquartile range (IQR).

FEMALE													
		21% O_2		15% O_2		12% O_2		9% O_2		7% O_2		21% O_2	
Oxygen-concentration		Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
V_T	+/+	8.37 (7.37–9.19)	0.345	8.01 (6.34–1.02)	0.753	9.53 (7.63–1.16)	0.674	7.76 (6.63–9.12)	1.0	5.89 (5.7–6.68)	0.172	6.87 (6.44–7.49)	0.248
	−/−	7.62 (7.13–8.06)		8.02 (7.25–1.04)		9.11 (7.4–1.04)		7.95 (7.49–8.86)		6.95 (6.85–8.17)		7.8 (7.16–8.4)	
f_R (beats/min)	+/+	242 (224.3–251.6)	0.345	217 (204.1–237.8)	0.753	220 (209.4–231.1)	0.074	200 (190.2–209.2)	0.208	188 (168.4–210.1)	0.462	237(225.7–242.6)	0.074
	−/−	240 (185.7–246)		217 (203.2–227.9)		203 (190.9–220.6)		185 (166.5–201.5)		178 (155.4–194.2)		211(198.8–225.9)	
MV (ml/g/min)	+/+	1.92 (1.8–2.12)	0.529	1.82 (1.42–2.2)	0.753	2.16 (1.75–2.44)	0.753	1.53 (1.29–1.94)	0.401	1.11 (0.98–1.38)	0.462	1.67 (1.48–1.8)	0.529
	−/−	1.86 (1.36–2.02)		1.93 (1.6–2.25)		2 (1.63–2.47)		1.51 (1.15–1.77)		1.27 (1.02–1.56)		1.71 (1.54–1.92)	
PEF (ml/g/s)	+/+	0.12 (0.11–0.13)	0.059	0.11 (0.091–0.14)	0.529	0.14 (0.11–0.16)	0.059	0.14 (0.12–0.17)	0.059	0.12 (0.11–0.14)	0.141	0.11 (0.1–0.12)	0.294
	−/−	0.09 (0.073–0.12)		0.11 (0.082–0.12)		0.11 (0.095–0.12)		0.11 (0.1–0.13)		0.11 (0.10–0.13)		0.102 (0.09–0.11)	
PIF	+/+	0.2 (0.16–0.23)	0.462	0.15 (0.13–0.17)	0.462	0.19 (0.15–0.22)	0.529	0.13 (0.10–0.15)	0.674	0.07 (0.065–0.09)	0.141	0.13 (0.11–0.16)	0.294
	−/−	0.17 (0.11–0.23)		0.19 (0.13–0.23)		0.18 (0.15–0.21)		0.14 (0.112–0.15)		0.10 (0.09–0.12)		0.15 (0.14–0.18)	
R_T	+/+	104 (93.9–113.4)	0.059	108 (103.2–119.9)	0.002	95 (86.7–99)	0.009	84.9 (77.2–89.9)	0.059	77.8 (68.2–89.1)	0.036	84.5 (77.6–89.7)	0.006
	−/−	118 (111.5–173.7)		136 (126.7–162.8)		122 (105.9–132)		98.8 (85.6–115.1)		97.2 (86.8–118.4)		105 (91.4–112.6)	

Table 3B
Respiratory parameters (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV); peak expiratory/inspiratory flow (PEF/PIF); expiration/inspiration time (T_E/T_I); relaxation time (R_T)) of male TASK-1$^{+/+}$ (n = 9) and TASK-1$^{-/-}$ (n = 11) mice under stepwise hypoxia: Genotype-related differences of both sexes are listed in the table (*p*-values: differences between genotypes). Data are presented as median and interquartile range (IQR).

MALE													
		21% O_2		15% O_2		12% O_2		9% O_2		7% O_2		21% O_2	
Oxygen-concentration		Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
V_T	+/+	6.71 (6.49–7.09)	0.053	6.07 (5.45–6.99)	0.017	6.68 (5.52–8.33)	0.02	5.29 (4.73–6.23)	0.007	4.88 (4.38–5.71)	0.002	8.65 (7.56–12.95)	0.011
	−/−	8.45 (7.05–12.14)		10.2 (8.51–16.17)		9.2 (7.83–17.15)		9.35 (7.23–17.1)		8.65 (7.56–12.95)		5.83 (5.21–6.34)	
f_R (beats/min)	+/+	206 (191.4–211.2)	0.037	209 (199.2–212.8)	0.970	209 (200.1–219.4)	0.003	184 (156.5–194.7)	0.184	159 (155.4–185.2)	0.184	239 (227.7–276.5)	0.102
	−/−	236 (207.6–249.4)		206 (189–229.03)		188 (175.7–198.1)		159 (153–174.6)		154 (141.1–186.1)		225 (207–243.9)	
MV (ml/g/min)	+/+	1.37 (1.24–1.47)	0.007	1.26 (1.18–1.63)	0.006	1.35 (1.14–1.72)	0.011	0.98 (0.83–1.17)	0.011	0.86 (0.75–1)	0.001	1.35 (1.26–1.67)	0.053
	−/−	2.1 (1.55–3.26)		2.31 (1.9–3.7)		1.82 (1.73–3.32)		1.51 (1.14–2.96)		1.55 (1.18–2.37)		2.03 (1.37–2.8)	
PEF (ml/g/s)	+/+	0.08 (0.07–0.092)	0.087	0.096 (0.08–0.10)	0.037	0.099 (0.09–0.11)	0.210	0.09 (0.085–0.12)	0.074	0.098 (0.09–0.11)	0.037	0.1 (0.081–0.106)	0.184
	−/−	0.12 (0.074–0.16)		0.13 (0.099–0.19)		0.12 (0.098–0.18)		0.14 (0.092–0.21)		0.16 (0.107–0.19)		0.13 (0.083–0.16)	
PIF	+/+	0.11 (0.082–0.12)	0.025	0.09 (0.071–0.16)	0.011	0.13 (0.087–0.14)	0.063	0.07 (0.064–0.09)	0.003	0.06 (0.05–0.06)	0.002	0.11 (0.11–0.15)	0.074
	−/−	0.21 (0.13–0.34)		0.19 (0.13–0.38)		0.17 (0.14–0.31)		0.13 (0.107–0.27)		0.11 (0.09–0.17)		0.19 (0.12–0.23)	
R_T	+/+	106 (101.4–118.6)	0.011	102 (97.3–103.7)	>.001	94 (83.4–99.4)	>.001	89 (79.3–95.5)	0.014	76 (61.4–99.4)	0.004	79 (68.2–80.8)	0.003
	−/−	126 (112.6–134)		143 (128.7–157)		133 (122.1–142.2)		123 (114.9–132.9)		120 (108.3–147.7)		103 (96.4–112.4)	

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Table 4
Respiratory parameters (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV); relaxation time (R_T)) of male TASK-1$^{+/+}$ (n = 9)/TASK-1$^{-/-}$ (n = 11) mice and female TASK-1$^{+/+}$ (n = 10)/TASK-1$^{-/-}$ (n = 10) mice under acute hyperoxic hypercapnia (5% CO_2/95% O_2): Genotype-related differences of both sexes are listed in the table (*p*-values: differences between genotypes). Data are presented as median and interquartile range (IQR).

		FEMALE						MALE					
		21% O_2		95% O_2/5% CO_2		21 % O_2		21% O_2		95% O_2/5% CO_2		21 % O_2	
		Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
V_T	+/+	10 (6.96–11.15)	0.762	16.75 (9.99–28.31	0.762	12 (6.10–15.9)	0.88	9.65 (8–10.91)	0.342	16.3 (8.6–19.11)	0.102	10.5 (5–11.6)	0.087
	−/−	8.93 (7.23–10.86)		13.9 (10.83–20.4)		8.6 (8.10–13.4)		12.9 (7.36–16.47)		25 (12.13–27.19)		13.5 (7.2–16.2)	
f_R (beats/min)	+/+	212 (209.6–225)	0.545	260 (232.9–299.3)	0.496	226 (218.4–237.1)	0.597	232 (207–248.1)	0.184	272 (236.1–282.2)	0.239	208 (194–221.7)	0.087
	−/−	201 (183–231.5)		283 (245–291.6)		219 (211–242)		237 (222–252)		279 (270.4-292.3)		231 (220–249.5)	
MV (ml/g/min)	+/+	1.78 (1.27–2.52)	0.940	3.15 (2.1–7.74)	0.597	2.35 (1.41–3.38)	0.88	2.19 (1.99–2.36)	0.184	4.5 (2.15–5.7)	0.025	2.09 (0.88–2.54)	0.002
	−/−	1.77 (1.37–2.44)		4.49 (2.69–5.96)		2.02 (1.78–3.15)		2.99 (2–3.78)		7.01 (3.42–8.04)		3.08 (1.78–3.64)	
R_T	+/+	118 (104.6–131)	0.013	66.9 (62.97–83.4)	0.650	127 (110.9–137.7)	0.173	105 (99.5–108.5)	0.102	70 (66.77–75.53)	0.160	115 (98–188.8)	0.087
	−/−	143 (126.7–176.1)		66.4 (60.18–81.1)		135 (124–157.9)		124 (101.5–179.2)		66 (62.68–69.35)		128 (109–148.8)	

Table 5A
Changes of respiratory parameters (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV) of male TASK-1$^{+/+}$ (n = 8) and TASK-1$^{-/-}$ (n = 8) mice under the influence of isoflurane: Genotype-related differences are listed in the table (p-values: differences between genotypes). Data are presented as median and interquartile range (IQR).

		0.2%		0.4%		0.6%		0.8%		1.0%		1.5%	
Isoflurane-concentration		Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
V_T	+/+	6.62 (5.24–6.95)	0.401	6.36 (5.19–7.66)	0.753	6.88 (5.4–8.98)	0.753	6.93 (5.91–7.87)	0.345	5.15 (4.43–5.72)	0.027	5.41 (4.25–6.15)	0.208
	−/−	7.25 (5.65–9.06)		7.8 (6.18–9.48)		8.13 (6.3–8.84)		7.75 (6.41–8.79)		6.3 (5.9–7.3)		6.29 (5.66–7.64)	
f_R (beats/min)	+/+	199 (187.1–241.2)	0.093	174 (162.3-200.7)	0.059	197 (172.6–206.2)	0.462	169 (165.2–177.2)	0.012	146 (139.7–150.8)	0.674	93 (86.1–106.5)	0.834
	−/−	252 (224.5–289.3)		158 (148.7–167.3)		181 (145.5–209.4)		157 (148.5–159.7)		139 (131.5–157.7)		91 (70.3–109)	
MV (ml/g/min)	+/+	1.29 (1.07–1.56)	0.009	1.16 (0.82–1.38)	0.345	1.31 (1.04–1.78)	0.753	1.28 (0.94–1.37)	0.916	0.76 (0.67–0.82)	0.009	0.46 (0.39–0.57)	0.021
	−/−	1.91 (1.57–2.02)		1.3 (1.04–1.49)		1.32 (1.18–1.68)		1.1 (0.98–1.35)		0.98 (0.9–1.07)		0.64 (0.57–0.81)	

Table 5B
Hypercapnic-induced respiratory response (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV) of male TASK-1$^{+/+}$ (n = 10) and TASK-1$^{-/-}$ (n = 10) mice under control condition (21% O_2) and the influence of isoflurane: Genotype-related differences are listed in the table (*p*-values: differences between genotypes). Data are presented as median and interquartile range (IQR).

		21% O_2		5% CO_2		21% O_2		1% Isoflurane		1 % Isoflurane/5% CO_2		1% Isoflurane	
		Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
V_T	+/+	6.03 (5.44–6.26)	0.082	9.99 (9.65–10.9)*	0.112	6.35 (5.83–6.81)	0.059	5.77 (5.27–6.08)	0.004	6.46 (6.42–7.9)#¶	0.013	5.26 (4.63–7.5)	0.174
	−/−	7.67 (6.21–11.07)		13.1 (11.4–16.4)*		8.13 (6.59–9.08)		8.51 (7.63–9.39)		9.81 (8.98–13.2)		6.39 (5.57–7.35)	
f_R (beats/min)	+/+	218 (193.2–230.1)	0.131	288 (269.6–307)*	0.112	245 (201.1–263.7)	0.364	168 (153.6–177.4)	0.496	130 (117.6–138)#¶	0.762	118 (101–126.3)	0.821
	−/−	228 (211–243.7)		257 (241.5–293)*		221 (199.8–241.2)		164 (145.7–182.1)		126 (116.8–139)#¶		110 (99.7–128.1)	
MV (ml/g/min)	+/+	1.35 (1.06–1.4)	0.041	3.01 (2.86–3.17)*	0.151	1.51 (1.19–1.71)	0.226	0.96 (0.91–1.05)	0.010	0.89 (0.82–0.92)#¶	0.041	0.66 (0.51–0.75)	0.257
	−/−	1.86 (1.41–2.39)		3.58 (2.97–4.31)**		2.04 (1.32–2.23)		1.34 (1.06–1.65)		1.3 (1.03–1.56)		0.74 (0.58–0.83)	

* p-value < 0.05 between control and hypercapnia of male TASK-1$^{+/+}$ mice. # p-value < 0.05 between isoflurane and hypercapnia/isoflurane of male TASK-1$^{+/+}$ mice.
** p-value < 0.05 between control and hypercapnia of male TASK-1$^{-/-}$ mice. ## p-value < 0.05 between isoflurane and hypercapnia/isoflurane of male TASK-1$^{-/-}$ mice.

Table 6
Respiratory parameters (tidal volume (V_T); respiratory frequency (f_R); minute volume (MV); peak expiratory/inspiratory flow (PEF/PIF); relaxation time (R_T)) of neonatal TASK-1$^{+/+}$ (n = 9–11) and TASK-1$^{-/-}$ (n = 3–8) mice under stepwise hypoxia: Genotype-related differences are listed in the table (*p*-values: differences between genotypes). Data are presented as median and interquartile range (IQR).

		21% O_2		15% O_2		12% O_2		11% O_2		21% O_2	
Oxygen-concentration		Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
V_T	+/+	10.93 (9.77–14)	0.026	9.22 (8.98–17.64)	0.008	8.41 (7.38–13.73)	0.159	7.84 (6.55–9.06)	0.463	6.69 (6.30–10.28)	0.128
	−/−	9.31 (8.77–10.7)		7.46 (6.19–8.83)		7.53 (5.02–8.99)		7.58 (5.26–8.35)		11.51 (8.18–12.08)	
f_R (beats/min)	+/+	292 (274.3–310.2)	<0.001	267 (249.9–297.1)	0.003	238 (219.7–272.6)	0.021	226 (196.5–258.8)	0.028	193 (164.6–224.9)	0.128
	−/−	210 (203.4–228.9)		197 (190.6–220.1)		186 (178.4–202.5)		189 (167.6–194.8)		168 (142.9–170.4)	
MV (ml/g/min)	+/+	3.24 (3.01–4.18)	<0.001	2.86 (2.46–5.12)	0.002	2.16 (1.96–3.04)	0.021	1.75 (1.65–1.85)	0.125	1.57 (1.19–1.73)	0.398
	−/−	2.26 (1.94–2.63)		1.58 (1.22–2.01)		1.71 (1.32–1.84)		1.5 (1–1.54)		1.93 (1.15–2.15)	
PEF (ml/g/s)	+/+	0.23 (0.15–0.27)	0.117	0.2 (0.14–0.3)	0.013	0.14 (0.11–0.2)	0.315	0.13 (0.09–0.14)	0.549	0.12 (0.099–0.152)	0.310
	−/−	0.18 (0.15–0.20)		0.11 (0.08–0.17)		0.13 (0.06–0.16)		0.11 (0.072–0.15)		0.14 (0.128–0.2)	
PIF	+/+	0.28 (0.25–0.37)	<0.001	0.23 (0.21–0.41)	<0.001	0.18 (0.16–0.26)	0.001	0.15 (0.13–0.15)	0.006	0.129 (0.12–0.16)	0.128
	−/−	0.16 (0.12–0.17)		0.11 (0.085–0.12)		0.1 (0.07–0.11)		0.1 (0.081–0.11)		0.11 (0.088–0.13)	
R_T	+/+	75.8 (67.1–77.7)	0.001	80.5 (67.9–89.7)	0.006	80.8 (65.9–95.7)	0.009	94.4 (72.4–104.5)	0.006	105.8 (93.73–139)	0.237
	−/−	99.8 (91.8–104.3)		115 (89.2–154.6)		124 (97.7–155)		155 (116.8–162.7)		139 (110.6–224.3)	

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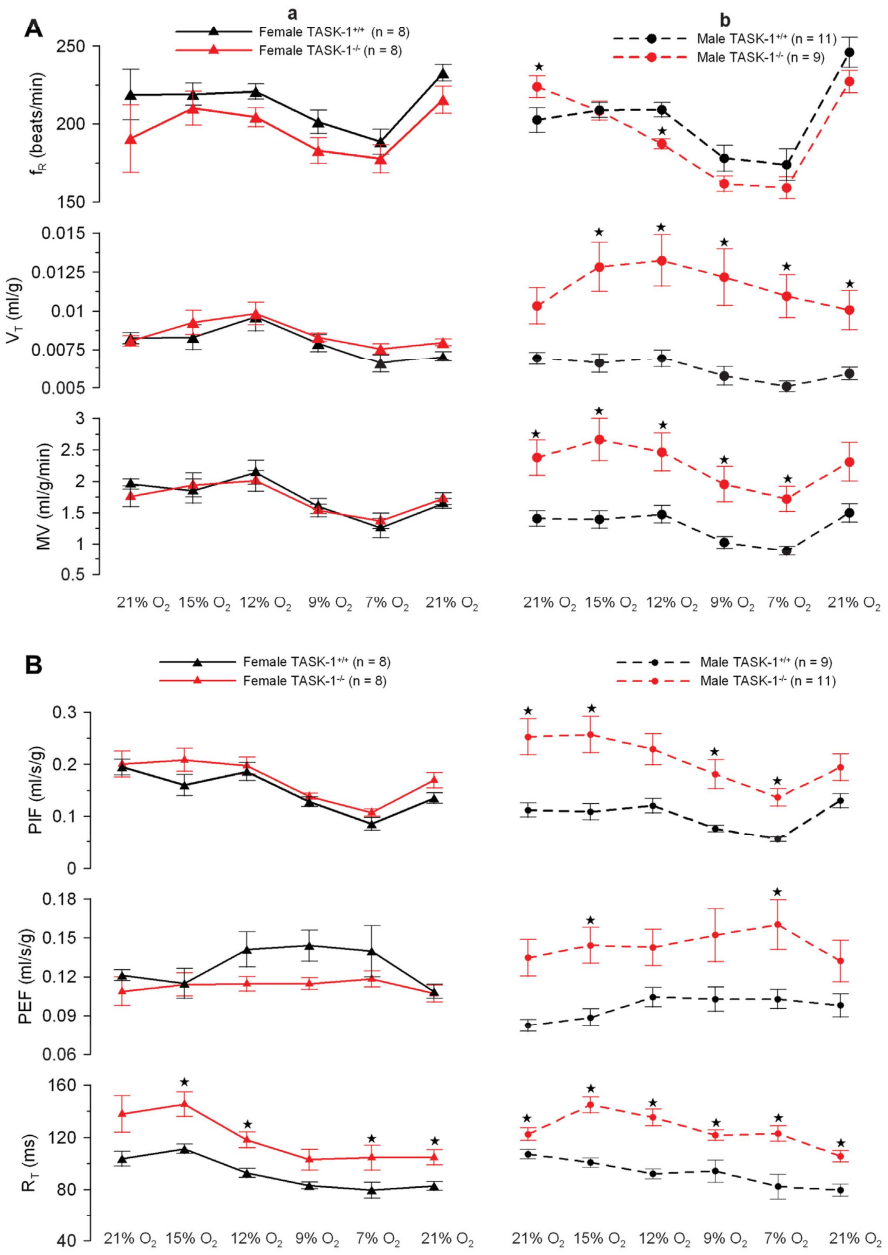

Fig. 2. A) Summary of the respiratory response to stepwise hypoxia for female and male TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice. Changes of respiratory frequency (f_R), tidal volume (V_T) and minute volume (MV) under stepwise hypoxia are depicted. Data are shown as mean ± SEM. $P < 0.05$: ⋆ significant differences between TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice of either female (column a) or male (column b) sex. B) Average changes of peak expiratory flow (PEF), peak inspiratory flow (PIF) and relaxation time (R_T) under step-wise hypoxia for female and male TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice. Female TASK-$1^{-/-}$ mice showed a similar PIF response and slightly restricted PEF increase under hypoxia. In male TASK-$1^{-/-}$ mice, PIF and PEF were increased under control and step-wise hypoxia. TASK-$1^{-/-}$ mice of both sexes exhibited prolonged R_T under control and hypoxia. Data are shown as mean ± SEM. $P < 0.05$: ⋆ between TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice of either female (column a) or male (column b) sex.

the expression of TASK-1 channels (Hao and Li, 2014). In this regard, transgenic female mice harboring high level of erythropoietin had a 4-fold increased level of estradiol, which compromised carotid body response to hypoxia (Gassmann et al., 2010). The compromised carotid body function in these transgenic mice could be induced by downregulation of TASK-1 in carotid bodies. Further-

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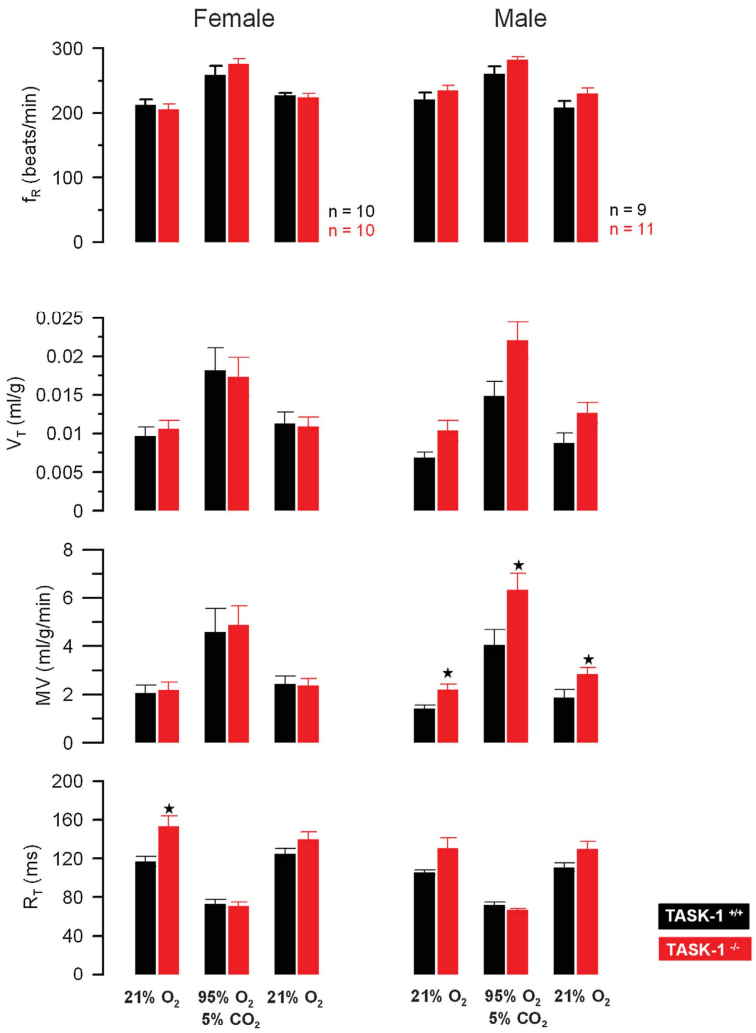

Fig. 3. Hypercapnic-hyperoxic-induced ventilatory response in female and male TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice. Average changes of respiratory frequency (f_R), tidal volume (V_T), minute volume (MV) and relaxation time (R_T) are depicted. Female TASK-$1^{-/-}$ mice showed the identical hypercapnia-stimulated ventilation like their wild type counterparts (5% CO_2/95% O_2). Under control and hypercapnia, male knockout mice displayed enhanced ventilation, but the relative increase of ventilation was identical in both genotypes (data not shown). Under CO_2 challenge, R_T differences disappeared in female TASK-$1^{-/-}$ mice. Data are shown as mean ± SEM. $P < 0.05$: ⋆ between TASK-$1^{+/+}$ and TASK-$1^{-/-}$ mice of either female or male sex.

more, it could be of interest to investigate respiration in castrated TASK-$1^{-/-}$ mice with or without additional supplementation of sex hormones.

4.2. Prolonged relaxation time in TASK-$1^{-/-}$ mice

Relaxation time (R_T) is a parameter of expiration kinetics and is defined as the time needed to exhale 64% of the tidal volume. Interestingly, TASK-1 gene inactivation induced a significant prolongation of the relaxation time (R_T) in adult and neonatal mice. The three-phased organization of respiratory cycle is highly conserved in mammalian species and is regulated by the respiratory rhythm generator in the brainstem. This concept distinguishes an augmenting inspiration (aug-I) from declining post-inspiratory (post-I) and augmenting expiratory phase (Richter and Smith, 2014). It is theoretically conceivable that the TASK-1 deletion might have prolonged the post-I phase by prolonging the activation of recurrent laryngeal nerve output. Such a mechanism could result in an extended expiration phase and thereby a longer R_T. Alternatively, the R_T prolongation could be caused by increased airway resistance, as described in patients with primary pulmonary arterial hypertension (PPAH) (Meyer et al., 2002). In fact, TASK-1 mutations seem to be one important cause for the developing PPAH (Ma et al., 2013; Girerd et al., 2014). However, in contrast to humans and rats (Antigny et al., 2016), TASK-1 null mice apparently don't develop PPAH (Manoury et al., 2011). Further studies will be needed to

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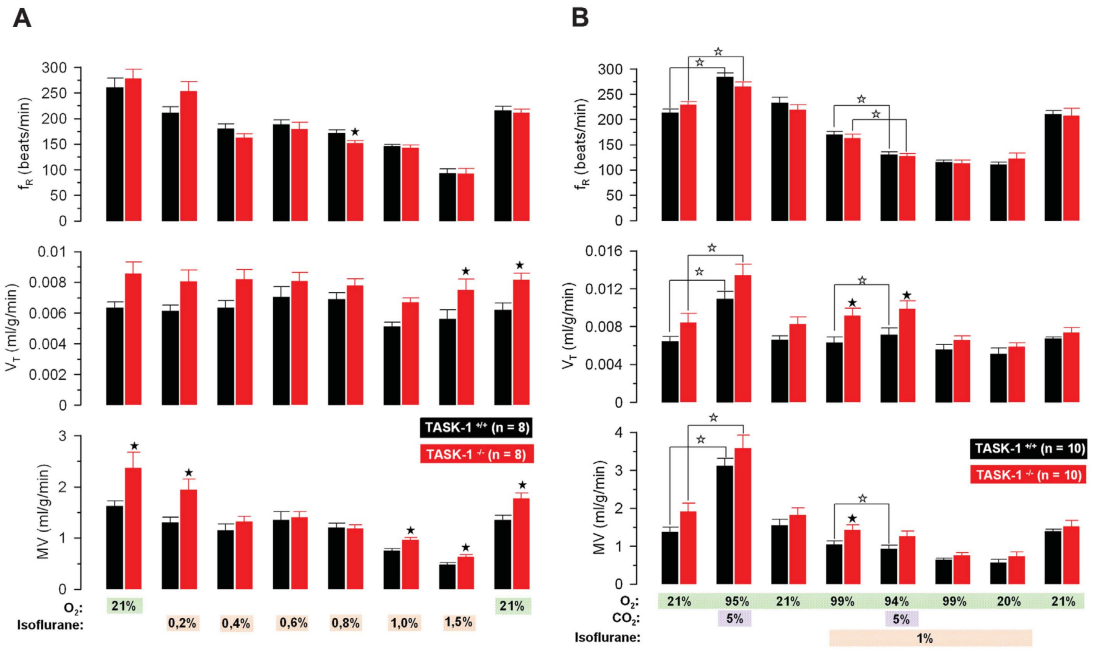

Fig. 4. Respiration in male TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice under the influence of isoflurane. Respiratory frequency (f_R), tidal volume (V_T) and minute volume (MV) are shown. **A:** Isoflurane induced concentration-dependently a continuous decline of f_R in both genotypes. Under low concentrations of isoflurane (0.4–0.8%) the increased ventilation in male TASK-1$^{-/-}$ (as observed at control conditions) disappeared. At higher concentrations of isoflurane (1–1.5%), it seemed that respiration was less suppressed in knockout animals. However, the relative suppression was not different in the both genotypes (data not shown). $P < 0.05$: ⋆ between male TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice. **B:** Effect of isoflurane (1%) on hypercapnia response isoflurane abolished the hypercapnia-induced increase in respiration in both genotypes. Data are shown as mean ± SEM. $P < 0.05$: ⋆ between male TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice; ☆ significant differences between control and experimental period (within a given genotype).

decipher the nature of the prolonged relaxation time in TASK-1$^{-/-}$ mice.

4.3. Increased tidal volume in TASK-1$^{-/-}$ mice

Besides the modified expiration kinetics, male TASK-1$^{-/-}$ mice also presented with an enhanced tidal volume (V_T). This feature, along with higher peak inspiratory and expiratory flows (PIF/PEF), an enhanced tidal volume (V_T) of male TASK-1$^{-/-}$ mice was a prominent finding of this study. The enhanced V_T, along with higher peak inspiratory and expiratory flows (PIF/PEF), could reflect a primary deficit of the neuronal control of respiration or it could be a consequence of increased need for oxygen, e.g. during increased activity (e.g. due to increased sympathetic tone) or due to reduced gas exchange in the lungs. The fact that TASK-1$^{-/-}$ mice did not display an increase of the respiratory frequency and constant higher V_T during longtime experiments over 24 h (data not shown) makes it more likely that the increase of V_T is reflecting modified respiratory control.

We would like to propose two possible mechanisms for the increased V_T in the knock out animals: i) affection in the Breuer-Hering reflex (Morschel and Dutschmann, 2009) and/or ii) modified function of respiratory brainstem neurons. TASK channels are expressed in many brainstem nuclei including the solitary tract (NTS) and the pre-Bötzinger nucleus (pre-BötC) (Bayliss et al., 2001; Washburn et al., 2002; Wang et al., 2008; Koizumi et al., 2010; Lazarenko et al., 2010a; Richter and Smith, 2014). The NTS is involved in transmission of afferent inputs from stretch receptors located in the lungs to the central respiratory rhythm generator. These inputs induce a stop of inspiration by late- and post-inspiratory synaptic inhibition (Richter and Smith, 2014). Thus, an incomplete transmission of the stretch receptor input could impair the Breuer-Hering reflex resulting in an enhanced V_T of adult male TASK-1$^{-/-}$ mice. Alternatively, deletion of the TASK-1 gene directly affect the function of respiratory nuclei such as pre-BötC, thereby altering the respiratory cycle and enhancing the depth of breathing.

4.4. Effects of hypoxia on respiration in adult TASK-1$^{-/-}$ mice

A study by Trapp et al. reported impaired hypoxia-induced ventilation in adult male TASK-1$^{-/-}$ mice (Trapp et al., 2008). Similar to this report, we also observed a reduced increase of the respiration frequency (f_R) in adult male TASK-1$^{-/-}$ mice. With regard to minute volume (MV), however, the respiratory response to hypoxia was not impaired in our study. It was suggested by others that TASK-1 or TASK-3 channel deletion in the carotid bodies (peripheral chemoreceptors) is compensated by yet unidentified mechanisms (Buckler, 2015). In addition, central brainstem mechanisms could contribute to the compensation of TASK-1 inactivation and, thereby, result in a not very obvious phenotype under hypoxic conditions.

4.5. Hypercapnia-stimulated respiration is not affected in TASK-1$^{-/-}$ mice

Central CO_2 chemo-sensitivity in mammals is mainly located in Phox2B-positive neurons of the retrotrapezoid nucleus (RTN), where TASK-2 channels and proton-activated receptor 4 (GPR4) are involved in CO_2 chemo-sensing (Gestreau et al., 2010; Wang et al., 2013; Kumar et al., 2015). It was suggested that neither

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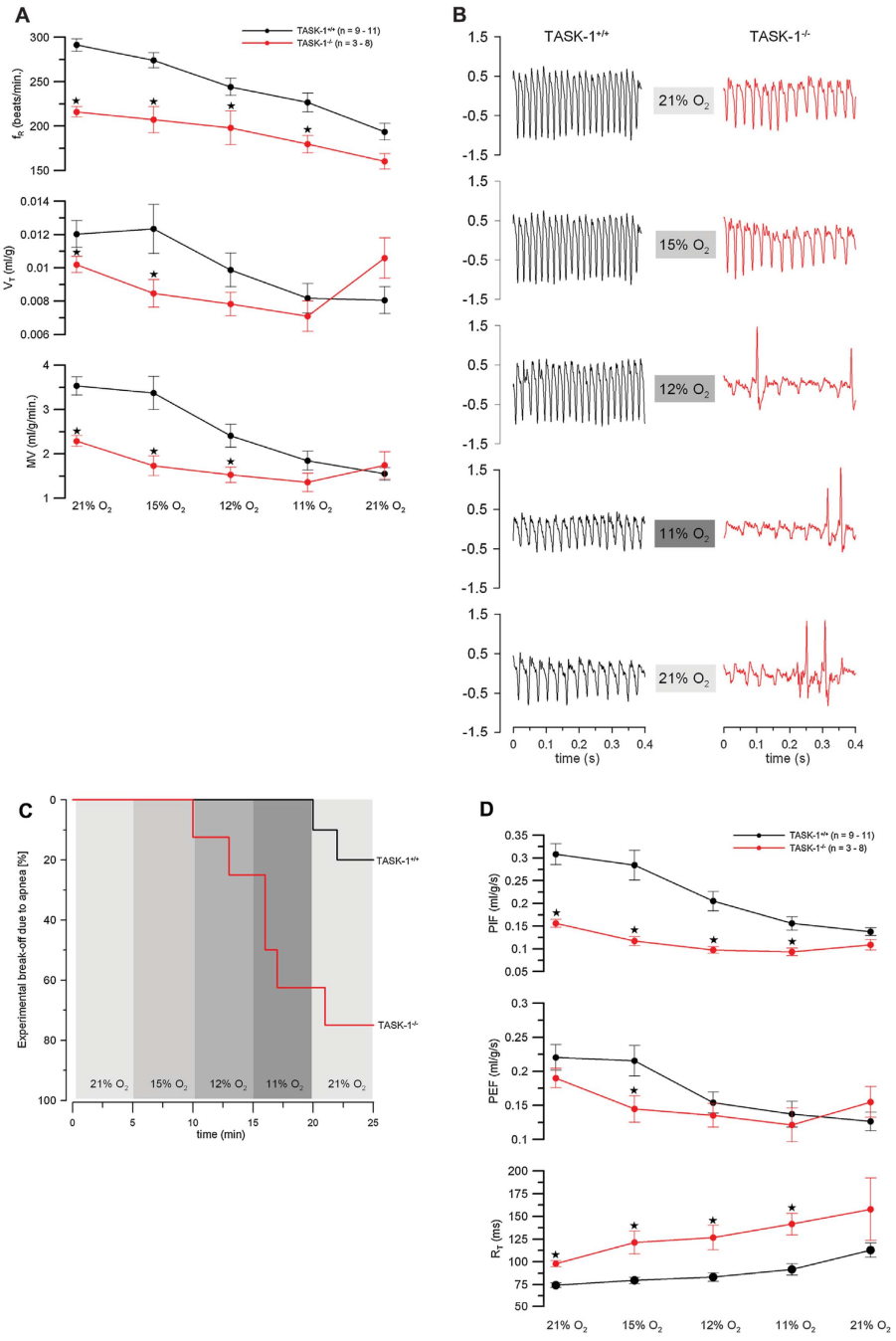

Fig. 5. Hypoxic-induced respiration in neonatal TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice of both sexes:.
A: Summary of the hypoxia-induced respiratory answer in neonatal TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice. Neonatal TASK-1$^{-/-}$ mice showed compromised ventilation. Data are shown as mean ± SEM. $P < 0.05$: ⋆ between neonatal TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice of both sexes.
B: Typical original respiratory trace from individual neonatal TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice under control- (21%) and hypoxia (15–11%).
C: Comparison of the occurrence of apnea (>3 s) between neonatal TASK-1$^{-/-}$ and TASK-1$^{+/+}$ mice during the hypoxia challenge, depicted as a Kaplan–Meier curve. Apnea was more frequently observed in neonatal TASK-1$^{-/-}$ mice ($p = 0.007$).

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TASK-1 nor TASK-3 channels are involved in central CO_2 chemosensing (Mulkey et al., 2007). In the present study, we exposed adult TASK-1$^{-/-}$ mice of both sex to CO_2 and found a normal respiratory response. In contrast to our data, Trapp et al. observed an impairment of CO_2-stimulated ventilation (3 and 5% CO_2 in normoxia) in male TASK-1$^{-/-}$ and TASK-1/3$^{-/-}$ mice (Trapp et al., 2008). This discrepancy might be related to the fact that we used, like Mulkey et al., a hyperoxic-hypercapnic gas mixture (hyperoxia might impair chemo-sensation of carotid bodies) instead of the normoxic-hypercapnic gas mixture used by Trapp and coworkers.

4.6. Isoflurane-induced respiratory depression in TASK-1$^{-/-}$ mice

TASK channels are activated by volatile anesthetics such as halothane and isoflurane and, thereby, hyperpolarize the plasma membrane resulting in silencing of neuronal activity. In a recent study, a mildly reduced halothane- and isoflurane-sensitivity in TASK-1$^{-/-}$ mice was found with regard to the immobilizing actions of these anesthetics (Lazarenko et al., 2010b). Here, we investigated the isoflurane-induced suppression of respiration in male TASK-1$^{-/-}$ mice. The isoflurane-induced respiratory depression was largely preserved in TASK-1$^{-/-}$ mice. However, a closer inspection revealed specific differences between knockout and wildtype mice: At low concentration of isoflurane (0.8%), TASK-1$^{-/-}$ mice appeared to be more sensitive and displayed a slightly increased suppression compared to wildtype mice. At concentrations of isoflurane in the therapeutic range (1 and 1.5%) relative respiratory suppression was similar in both genotypes. A possible explanation for the small differences in the isoflurane-induced respiratory depression could be the fact that TASK-3 homomeric and TASK-1/TASK-3 heterodimeric channels are more sensitive to isoflurane, whereas TASK-1 homomeric channels are relatively unaffected (Berg et al., 2004). Furthermore, inhalation anesthetics act partially through gamma-aminobutyric acid ($GABA_A$) receptor activation. Therefore the proposed up-regulation of $GABA_A$ receptor function in TASK-1 KO mice might mask the role of TASK-1 channel as a target for inhalation anesthetics (Linden et al., 2008)

Since the potency of isoflurane to cause respiratory suppression was slightly enhanced in TASK-1$^{-/-}$ mice, we tested if this phenotype was caused by a modified sensitivity to CO_2 and hypoxia in the presence of isoflurane. Interestingly, the CO_2 and hypoxia response was blunted in both genotypes in the presence of isoflurane indicating the isoflurane-induced activation of TASK-1 is not an indispensable component for its suppressive effect on the CO_2- and hypoxia response.

4.7. Respiratory phenotype of neonatal TASK-1$^{-/-}$ mice

In contrast to the mild hypoxia phenotype of adult mice, neonatal TASK-1$^{-/-}$ mice showed a pronounced respiratory phenotype (Fig. 5A–D). Neonatal TASK-1$^{-/-}$ mice exhibited a reduction of ventilation of some 35% under control conditions, which is in the similar range of the reduction observed in animals after "surgical ablation" of the carotid bodies (Rodman et al., 2001; Izumizaki et al., 2004). During hypoxia, neonatal TASK-1$^{-/-}$ mice showed significant more and longer periods of apnea and in 6 out of 8 animals the hypoxia period needed to be stopped ahead of schedule (Fig. 5B). What are possible mechanisms for the unstable respiration of neonatal TASK-1$^{-/-}$ mice? One might speculate that TASK-1 deletion in neonatal mice resulted in a heavily impaired carotid body function (peripheral chemoreception) or impaired central chemoreception and/or rhythm generation. With age, compensation takes place yielding stabilization of respiration (Wasicko et al., 2006). At present, it is not possible to distinguish between a central or peripheral origin of the impairment of respiration of neonatal TASK-1$^{-/-}$ mice. It is possible that TASK-1 has a similarly important role in human neonates. In this respect, TASK-1 channel down-regulation in carotid bodies of neonatal mice chronically treated with oxygen was assumed to contribute to an impaired hypoxia-response (Kim et al., 2012b). This impaired hypoxia-response can also be seen in humans treated for a longer period with oxygen during the neonatal period (Bouferrache et al., 2000; Bates et al., 2014).

The negative screening result for mutations in the human TASK-1 gene in 155 SIDS cases speaks against a prominent role of TASK-1 in SIDS. On the other hand, SIDS is rare in neonates and most frequently in infants of 2–3 month (Kinney and Thach, 2009). Therefore, TASK-1 channels still could be of relevance for stabilizing respiration in neonates under hypoxic conditions.

5. Conclusion

The present study provides evidence for a sex- and age-dependent respiratory phenotype in TASK-1$^{-/-}$ mice. Whereas adult female TASK-1$^{-/-}$ mice had a virtually normal respiratory phenotype, except for an increased relaxation time, adult male TASK-1$^{-/-}$ mice exhibited enhanced ventilation that was mainly due to an increased tidal volume. The respiratory responses to hypercapnia and hypoxia were largely preserved. In neonatal TASK-1$^{-/-}$ mice, respiratory drive appeared reduced and knockout animals were more prone to experience periods of apnea, especially when exposed to hypoxia. Further studies will be needed to investigate a potential role of TASK-1 channels for respiration of humans, especially for establishing the respiratory drive in neonates. Nevertheless a prominent role of impaired TASK-1 channel function in sudden infant death syndrome (SIDS) has been excluded by a negative screening result for TASK-1 mutations in 155 SIDS cases.

Conflict of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resp.2016.11.005.

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D: Average changes of peak expiratory flow (PEF), peak inspiratory flow (PIF) and relaxation time (R_T) under step-wise hypoxia in neonatal TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice of both sexes. Impaired PIF in the neonatal TASK-1$^{-/-}$ mice under control and hypoxia indicated reduced breath depth. The R_T was prolonged in neonatal TASK-1$^{-/-}$ mice, too. $P < 0.05$: ⋆ between neonatal TASK-1$^{+/+}$ and TASK-1$^{-/-}$ mice of both sexes.

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PUBLICATIONS

2017 **J. Neubauer,** M. R. Lecca, G. Russo, C. Bartsch, A. Medeiros-Domingo, W. Berger, C. Haas (2016) Post-mortem whole exome analysis in large sudden infant death syndrome cohort with a focus on cardiovascular and metabolic genetic diseases. *Eur J Human Genet*

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2017 **J. Neubauer,** R. Lecca, G. Russo, C. Bartsch, A. Medeiros-Domingo, W. Berger, C. Haas. Molecular autopsy in SIDS and SUD. Sommertagung der Schweizerischen Gesellschaft für Rechtsmedizin SGRM. Aarau, Switzerland, 09.06.-10.06.2017. Oral presentation

2016 **J. Neubauer,** R. Lecca, G. Russo, C. Bartsch, A. Medeiros-Domingo, W. Berger, C. Haas. Whole exome sequencing in 161 sudden infant death syndrome (SIDS) cases with a focus on cardiovascular and metabolic genetic diseases. American Society of Human Genetics ASHG2016, Vancouver, Canada, 18.10.-22.10.2016. Poster presentation

J. Neubauer, R. Lecca, G. Russo, C. Bartsch, A. Medeiros-Domingo, W. Berger, C. Haas. Whole-exome sequencing in 161 sudden infant death syndrome (SIDS) cases identifies potentially disease-associated variants in one third of our cohort. 27. Jahrestagung der Deutschen Gesellschaft für Humangenetik, Lübeck, Germany, 16.03.-18.03.2016. Oral presentation.

2014 **J. Studer**, C. Haas. Genetic mechanisms involved in sudden infant death syndrome (SIDS): early experience with exome approach. Sudden Cardiac Death Symposium, Bern, Switzerland, 06.11.-07.11.2014. Oral presentation

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J. Studer, C. Bartsch, C.Haas. Genetic risk factors in sudden infant death syndrome (SIDS). Sommertagung der Schweizerischen Gesellschaft für Rechtsmedizin SGRM. Zermatt, Switzerland, 14.06.-15.06.2013. Oral presentation

DECLARATION

I declare that the present thesis was composed by myself and the enclosed experimental work was performed on my own as indicated.
This dissertation has not been submitted for any other degree or professional qualification except as specified.

Jacqueline Neubauer